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(51) International Patent Classification ⁴ : C07H 15/12, C07K 13/00 A61K 37/48, 39/00	A1	(11) International Publication Number: WO 90/01035 (43) International Publication Date: 8 February 1990 (08.02.90)
(21) International Application Number: PCT/US89/03267 (22) International Filing Date: 24 July 1989 (24.07.89) (30) Priority data: 223,270 23 July 1988 (23.07.88) US 283,739 13 December 1988 (13.12.88) US 334,304 27 April 1989 (27.04.89) US (71) Applicant: THE UNITED STATES OF AMERICA, represented by THE SECRETARY, UNITED STATES DEPARTMENT OF COMMERCE [US/US]; Washington, DC 20231 (US). (72) Inventors: BERGER, Edward, A. ; 5820 Inman Park Circle, Rockville, MD 20852 (US). MOSS, Bernard ; 10301 Dickens Court, Bethesda, MD 20814 (US). FUERST, Thomas, R. ; 604 Paradise Ct., Gaithersburg, MD 20877 (US). MIZUKAMI, Tamio ; 106 Weymouth Street, Bethesda, MD 20814 (US). PASTAN, Ira, H. ; 11710 Beall Mountain Rd., Potomac, MD 20854 (US). FITZGERALD, David, J., P. ;		1731 Ladd Street, Silver Spring, MD 20902 (US). CHAUDHARY, Vijay, K. ; 5525 Halpine Place, Apt. , 101, Rockville, MD 20851 (US). (74) Agents: OLIFF, James, A. et al.; Oliff & Berridge, 277 S. Washington Street, Alexandria, VA 22314 (US). (81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent). Published <i>With international search report.</i>
(54) Title: CYTOTOXIC AGENT AGAINST SPECIFIC VIRUS INFECTION (57) Abstract A chimeric gene directing the synthesis of hybrid recombinant fusion protein in a suitable expression vector has been constructed. The fusion protein possesses the property of selective cytotoxicity against specific virus-infected cells. A CD4(178)-PE40 hybrid fusion protein has been made for selectively killing HIV-infected cells. A recombinant, soluble, truncated form of CD4 containing the active binding site for human immunodeficiency virus is provided. Novel hybrid proteins containing human CD4 sequences linked to human immunoglobulin constant regions to inhibit HIV infection are described.		

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Serial No.: 10/804,802
Filed : March 19, 2004
Exhibit 6

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CYTOTOXIC AGENT AGAINST SPECIFIC VIRUS INFECTIONTechnical Field

The present invention is related generally to the control of viral infection. More particularly, the present invention is related to the construction of a chimeric gene expressing a recombinant fusion protein which selectively kills specific virus-infected cells; to a recombinant soluble truncated form of CD4 containing the active binding site for human immunodeficiency virus; and to a multivalent product having substantially long half-life, bonding avidity and the capacity to direct components of the native immune system to kill HIV infected cells or HIV virions in vivo relative to monomeric forms. A hybrid fusion protein having selective cytotoxicity against HIV infected cells has been made.

Background of Invention

It is estimated that in the absence of effective therapy, most, if not all, individuals infected with human immunodeficiency virus (HIV) will develop acquired immune deficiency syndrome (AIDS) and ultimately succumb to a combination of opportunistic microbial infections and malignancies. It is further estimated that without an effective vaccine, the number of infected individuals is likely to increase substantially.

Anti-viral agents, immunomodulators and inhibitors of specific HIV functions are being tested as potential treatments to alleviate the high morbidity and mortality related to AIDS. However, a potent cytotoxic agent targeted to selectively kill HIV-infected cells has not heretofore been developed.

CD4 derivatives are uniquely suitable for AIDS treatment, since divergent strains of HIV-1 and HIV-2 all infect human T lymphocytes by binding to surface CD4. A recent report describes recombinant proteins containing soluble CD4 linked to human immunoglobulin heavy constant region sequences (Capon et al. 1989. Nature (London) 337, 525-531). However, the molecules did not bind complement component C₈, and no activity against HIV-

- 2 -

infected cells or virions was presented. The hybrid proteins of the present invention have distinctive properties not heretofore known or described.

5 CD4 is an integral membrane glycoprotein of human helper T lymphocytes that serves as an essential component of the receptor for the human immunodeficiency virus (HIV) (Popovic et al. 1984. Clin. Res. 33, 560A abstr.; and Maddon et al. 1986. Cell 47, 333-348), the
10 causative agent of acquired immunodeficiency syndrome. HIV binding and fusion with the cell are mediated by specific interaction between the external subunit of the viral envelope glycoprotein (gp120) and CD4 on the target cell surface [McDougal et al. 1986, Science 231, 382-385; Sodroski et al. 1986. Nature (London) 322, 470-474; and
15 Lundin et al. 1987. J. Immunol. Methods 97, 93-100].

Recent studies have yielded considerable insight into the structure of the CD4 molecule and its counterpart from other species. The primary sequence deduced from the human cDNA (Maddon et al. 1985. Cell 42, 93-104) and from N-terminal protein sequence analysis
20 (Fisher et al, 1988, Nature, 331: 76-78) indicates that the processed molecule is 433 amino acid residues in length, with a long N-terminal extracellular region followed by a transmembrane segment and a C-terminal
25 cytoplasmic tail. The external region contains an N-terminal domain of 100 amino acid residues that shares striking sequence homology and secondary structural features with the immunoglobulin light chain variable domain.

30 The remainder of the external region (270 residues) appears to be composed of three additional domains that also display structural relationships to the immunoglobulin family (Clark et al. 1987. Proc. Natl. Acad. Sci. USA 84, 1649-1653). The finding of introns
35 separating the coding sequences for these regions in the CD4 gene (Littman, D.R. 1987. Annu. Rev. Immunol. 5, 561-584) supports this notion of structural and possibly

- 3 -

functional domains. Of particular interest is the existence of conserved pairs of cysteine residues that probably form intradomain disulfide bonds within the first, second, and fourth external domains (Classon et al. 1986. Proc. Natl. Acad. Sci. USA 83, 4499-4503). These structural features deduced from cDNA sequencing have been complemented by epitope analyses using panels of anti-CD4 monoclonal antibodies (mAbs). Such studies have shed light on the topographic relationships of the various epitopes to one another, to the HIV-binding site, and to the cell membrane (Lundin et al, supra; Rao et al. 1983. Cell. Immunol. 80, 310-319; and Sattentau et al. 1986. Science 234, 1120-1123). However, the region(s) of the CD4 molecule involved in binding to the HIV envelope glycoprotein heretofore have not been identified.

SUMMARY OF THE INVENTION

It is, therefore, an object of the present invention to provide a chimeric gene which directs the synthesis, in a suitable expression vector, of a hybrid protein comprising a virus binding region from a cellular receptor sequence linked to a protein toxin sequence containing a region essential for cell toxicity.

It is a further object of the present invention to provide an isolated, substantially pure fusion protein comprising the HIV binding portion of the human CD4 molecule and active regions of Pseudomonas exotoxin A.

It is another object of the present invention to provide an anti-HIV composition comprising a polypeptide molecule made of about 180 amino acid residues representing the first two immunoglobulin-like domain of CD4 and having immunological and functional properties of an active HIV-binding site.

It is also an object of the present invention to provide multimeric human CD4-immunoglobulin recombinant proteins which selectively bind to gp120 on the surface of HIV-infected cells with higher affinity than monomeric soluble CD4 and which can direct components of

- 4 -

the immune system (complement and ADCC) to fight HIV infection.

5 It is yet another object of the present invention to provide an anti-HIV reagent containing only human sequences so that heat immune responses, which usually occur with foreign proteins, are minimized when the reagent of the present invention is administered to humans.

10 It is a further object of the present invention to provide a method of killing HIV infected cells or neutralizing HIV activity through the hybrid proteins of the present invention.

15 And it is also an object of the present invention to provide a method of inhibiting HIV infection, comprising administering to an HIV-infected host, an effective amount of the truncated CD4 molecule of the present invention to inhibit the infection of host cells by HIV.

20 It is another object of the present invention to provide a method of controlling AIDS virus infection, comprising contacting HIV-infected cells with effective amounts of the fusion protein of the present invention to selectively kill the HIV-infected cells.

25 Other objects and advantages of the present invention will become evident from the following detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

30 These and other objects, features and many of the attendant advantages of the invention will be better understood upon a reading of the following detailed description when considered in connection with the accompanying drawings wherein:

35 Figure 1 is a schematic representation of the plasmid used for expressing CD4(178)-PE40.

Figure 2 shows the results of column chromatography (2A) and gel electrophoresis (2B) of the CD4(178)-PE40;

- 5 -

Figure 3 demonstrates the binding of CD4(178)-PE40 to HIV envelope protein gp120 by (3A) coprecipitation technique and (3B, 3C, 3D and 3E) by immunofluorescence microscopy; and

5 Figure 4 shows the selective cytotoxic effect of CD4(178)-PE40 on cells expressing HIV-1 envelope glycoprotein: 4A. Cells expressing the HIV-1 envelope glycoprotein encoded by a recombinant vaccinia virus. Closed symbols represent cells infected with vPE-16, a
10 vaccinia recombinant containing the HIV-1 gp160 gene linked to the vaccinia 7.5 K promoter, inserted within the thymidine kinase locus. Open symbols represent cells infected with a control vaccinia recombinant, vTF7-3, which contains the bacteriophage T7 RNA polymerase gene
15 also linked to the vaccinia 7.5 K promoter and inserted within the thymidine kinase locus. The toxin preparations used were: Δ , \blacktriangle PE, O, \bullet PE40, \square , \blacksquare CD4(178)-PE40. 4B. Cells chronically infected with HIV-1. Closed symbols represent the 8E5 human T-cell line which contains a single integrated copy of the HIV-1 (LAV) genome. The virions produced are non-infectious due to a premature chain termination mutation in the reverse transcriptase gene. Open symbols represent the parental non-infected A3.01 cell line. The toxin preparation used
20 were: Δ , \blacktriangle PE, O, \bullet PE40, \square , \blacksquare CD4(178-PE40).

Figure 5 shows schematic construction of plasmid pCD4_f. Plasmid pTK7-5 contains the bacteriophage T7 gene 10 promoter (P_{T7}) and the T7 terminator (T_{T7}) separated by a unique BamHI site. This region is flanked
30 by vaccinia virus thymidine kinase left (TK_L) and right (TK_R) sequences. An adapter made by using two partially complementary synthetic oligonucleotides (GATCGAATTCAGGC-CTAATTAATTAAGTCGAC and GATCGTCGACTTAATTAATTAGGCCTGAATTC) was ligated into the BamHI site of pTF7-5 by using the
35 BamHI 5' overhangs of the adapter. The BamHI site is destroyed in the desired recombinants. Hence the reaction mixture was digested with BamHI to linearize recir-

- 6 -

cularized plasmids lacking the insert, and ampicillin-resistant transformants were screened by restriction mapping to identify those containing the insert in the desired orientation. Plasmid pEB-2 contains unique sites for EcoRI and Stu I, followed by a universal termination sequence (UTS) providing termination codons in all three reading frames, followed by a unique Sal I site. The desired CD4 DNA fragment was obtained by digesting pCD4-GEM4 with Nhe I (which cleaves the plasmid at a unique site at nucleotide 678 of the CD4 cDNA sequence), filling in the staggered end with the Klenow fragment of DNA polymerase I and dNTPs, and then digesting with EcoRI. The resulting 0.68-kilobase EcoRI-Nhe I fragment, which contains the ATG initiation codon of CD4, was force-cloned into pEB-2, which had been digested with EcoRI and Stu I. The Stu I site in the vector is destroyed in the recombinant. Hence the reaction mixture was digested with Stu I, and pCD4, was identified by restriction mapping of the ampicillin-resistant transformants. DNA sequence analysis of pCD4 indicated that the last amino acid of the CD4 sequence is the leucine at position 177 of the processed translation product, and that two additional C-terminal residues, proline and asparagine, are derived from translation of a portion of the UTS sequence in the vector.

Figure 6 shows the results of analysis of the metabolically labeled transient expression products. Transient metabolic labeling reactions were performed in cells infected with vTF7-3 and transfected with either plasmid pEB-2 or pCD4_f, and the media were collected. For immunoprecipitation, reaction mixes were prepared containing 1.1 ml of transfection medium, 0.99 ml of protease inhibitor buffer, and 0.11 ml of 20% (vol/vol) Nonidet P-40. The samples were cleared by incubation with 0.13 ml of a 20% (vol/vol) suspension of protein A-agarose for 1 hr at 4°C; this was followed by centrifugation. To the supernatants were added 2 ug of each of the

- 7 -

following murine mAbs: MT151, OKT4, and OKT4A (all are IgG₂). After overnight incubation, 0.05 ml of 20% suspension of protein A-agarose was added, and the incubations were continued for 3 hr at 4°C on a rotator. The samples were centrifuged and pellet and supernatant fractions were saved. The pellets were washed and treated with 0.1 ml of sample buffer containing 8 M urea, and 0.09-ml aliquots were analyzed by gel electrophoresis. (6A) Analysis on 12% gels of the total medium fractions (0.05 ml) and the corresponding supernatant fractions remaining after radioimmunoprecipitation. Lanes 1 and 2, total medium of cells transfected with pCD4_f (lane 1) and the corresponding immunoprecipitation supernatant (lane 2); lanes 3 and 4, total medium of cells transfected with pEB-2 (lane 3) and the corresponding immunoprecipitation supernatant (lane 4). (6B) Analysis on 15% gels of immunoprecipitates from medium of cells transfected with pCD4_f (lane 5) and cells transfected with pEB-2 (lane 6). The arrow on the left indicates the position of the CD4 fragment band, and the numbers in the center represent molecular weight markers (expressed as $M_f \times 10^{-3}$).

Figure 7 shows the results of epitope analysis of the CD4 fragment. Cells were metabolically labeled with ³⁵S-cysteine after infection with vTF7-3 and transfection with pCD4_f. The medium was collected. A mixture was prepared containing 0.55 ml of this transfection medium, 0.55 ml of protease inhibitor buffer, 0.07 ml of 20% (vol/vol) Nonidet P-40, and 1.58 ml of phosphate-buffered saline containing 0.02% (wt/vol) sodium azide. The mixture was cleared with 0.28 ml of a 20% suspension of protein A-agarose as described for Fig. 2, and 0.27 ml aliquots (representing 0.05 ml of initial transfection medium) were treated with 1 ug of the indicated mAbs. After overnight incubation at 4°C, each sample received 0.05 ml of a 20% (vol/vol) suspension of protein A-agarose that had been previously coated with saturating amounts of rabbit antiserum to mouse IgG, thereby circum-

- 8 -

venting potential problems associated with antibodies of different subclasses. Samples were incubated on a rotator for 4 hr at 4°C, and the pellets were collected and washed. They were dissolved in 0.09 ml of sample
5 buffer containing 8 M urea and applied to 12% polyacrylamide gels. Lane 1, total transfection medium (0.05 ml); lane 2, immunoprecipitate obtained with a control mAb (2E12.1). Immunoprecipitates were obtained with a battery of anti-CD4 mAbs: lane 3, MT151; lane 4, Leu3A;
10 lane 5, OKT4; lane 6, OKT4A; lane 7, OKT4B; lane 8, OKT4C; lane 9, OKT4D; lane 10, OKT4E; lane 11, OKT4F. Molecular weight markers are shown on the left (expressed as $M_f \times 10^{-3}$).

Figure 8 demonstrates the interaction of the
15 CD4 fragment with gp120. Medium from cells metabolically labeled after infection with vTF7-3 and transfection with pCD4 was used as the source of the CD4 fragment. Media from unlabeled or metabolically labeled cells doubly
20 infected with vTF7-3 plus vPE-6 served as the source of the gp120. Molecular weight markers are shown on the left (expressed as $M_f \times 10^{-3}$).

(8A) Coprecipitation of the CD4 fragment and gp120 by using an anti-gp120 mAb. Initial reaction mix-
tures contained 0.075 ml of each of the indicated media,
25 0.05 ml of protease inhibitor buffer, and 0.01 ml of 20% (vol/vol) Nonidet P-40. In the case of the double and single virus infection media, mixtures containing 1 part
30 labeled and 19 parts corresponding unlabeled media were used. After preincubation for 4 hr at room temperature (about 22°-25°C), 0.14-ml aliquots were removed and the indicated MAbs were added (anti-gp120, 0.1 ml of
hybridoma 902 supernatant; anti-CD4, 1 ug of OKT4A). The immune complexes were collected with protein A-agarose
35 IgG and processed for electrophoresis on 12% gels as described in the legend to Fig. 7.

Lane 1, total reaction mixture from the incuba-

- 9 -

tion containing the transfection medium and the vTF7-3 plus vPE-6 double infection medium. The immune precipitates were obtained from reactions containing the following additions: lane 2, transfection medium, vTF7-3 plus
5 vPE-G double infection medium, no antibody; lane 3, normal medium containing 2.5% fetal bovine serum, vTF7-3 plus vPE-6 double infection medium, anti-gp120; lane 4, transfection medium, normal medium containing 2.5% fetal bovine serum, anti-gp120; lane 5, transfection medium,
10 vTF7-3 single infection medium, anti-gp120; lane 6, transfection medium, vTF7-3 plus vPE-6 double infection medium, anti-gp120; lane 7, transfection medium, normal medium containing 2.5% fetal bovine serum, anti-CD4.

(8B) gp120 inhibition of immunoprecipitation of
15 the CD4 fragment by an anti-CD4 mAb. Initial reaction mixtures contained 0.05 ml of metabolically labeled transfection medium, 0.05 ml of protease inhibitor buffer, 0.01 ml of 20% Nonidet P-40, and 0.04 ml of phosphate-buffered saline containing 0.02% (wt/vol)
20 sodium azide. The mixtures were supplemented with the unlabeled media indicated below and allowed to incubate at room temperature for 5 hr. Ten nanograms of anti-CD4 mAb OKT4A was added, and the incubations were continued overnight at 4°C. Immune complexes were collected and
25 processed as described in the legend to Fig. 6 and electrophoresed on 12% gels. The supplementary media added during the initial incubation were as follows: lane 8, normal medium containing 2.5% fetal bovine serum; lane 9, vTF7-3 single infection medium; lane 10, vTF7-3 plus vPE-
30 6 double infection medium.

Figure 9 is a schematic representation of CD4-immunoglobulin hybrid proteins which were made in this invention.

Figure 10 shows the construction scheme of the
35 intermediate plasmid, pCD4CH1 which was used for the construction of the following plasmids for the expression of the hybrid proteins.

- 10 -

Figure 11 shows the construction scheme of pCD4ITM10 and pCD4ITM10G which express CD4(109)CH.

Figure 12 shows the construction scheme of pCD4ITM20 and pCD4ITM20G which express CD4(178)CH.

5 Figure 13 shows the construction scheme of pCD4ITM30 and pCD4ITM30G which express CD4(372)CH.

Figure 14 shows the construction scheme of pCD4ITM40G which expresses CD4(181)CL.

10 Figure 15 shows the expression of CD4(109)CH, CD4(178)CH, and CD4(372)CH in CV-1 cells. The immunocomplexes were analyzed by SDS-polyacrylamide gel (10%) electrophoresis in a reducing condition in the presence of 5% beta-mercaptoethanol. Lanes 1, 3, 5, 7 and 9 represent the analysis of the culture media obtained by
15 transfection of CV-1 cells by pEB2 (Berger et al. 1988, Proc. Natl. Acad. Sci. USA 85, 2357-2361), pCD4LTM1. (Mizukami et al. 1988, Proc. Natl. Acad. Sci. USA 85, 9273-9277), pCD4ITM10, pCD4ITM20, and pCD4ITM30, respectively. Lanes 2, 4, 6, 8 and 10 represent the analysis
20 of the extracts of cells obtained by transfection of CV-1 cells by pEB2, pCD4LTM1, pCD4ITM10, pCD4ITM20, and pCD4ITM30, respectively. Molecular weight markers are shown on the right (expressed as kilodalton).

25 Figure 16 shows the pattern of CD4(109)CH, CD4(178)CH, and CD4(372)CH expressed in CV-1 cells analyzed by SDS-polyacrylamide gels (7.5%) in a non-reducing condition. Lanes 1, 2, 3, 4 and 5 represent the analysis of the extracts of cells obtained by transfection of CV-1 cells by pEB2, pCD4LTM1, pCD4ITM10,
30 pCD4ITM20, and pCD4ITM30, respectively. Lanes 6, 7, 8, 9 and 10 represent the analysis of the culture media obtained by transfection of CV-1 cells by pEB2, pCD4LTM1, pCD4ITM10, pCD4ITM20, and pCD4ITM30, respectively.

35 Figure 17 shows the coexpression of CD4(178)CH with CD4(181)CL in CV-1 cells. The immunocomplexes were analyzed by SDS-polyacrylamide gel (7.5%) electrophoresis in a reducing condition (lanes 1-4) or a non-reducing

- 11 -

condition (lanes 5-8). Lanes 1 and 5 represent the analysis of the culture media of pTM3-transfected cells, lanes 2 and 6 for the culture media of pCD4ITM20G-transfected cells, lanes 3 and 7 for the culture media of pCD4ITM40G-transfected cells, and lanes 4 and 8 for the culture media of pCD4ITM20G and pCD48ITM40G doubly-transfected cells.

Figure 18 shows the schematic model structure of CD4(176)CH and CD4(181)CL tetrameric complex.

Figure 19 shows the expression of CD4(109)CH, CD4(178)CH, and CD4(372)CH in RPMI8226 cells. The immunocomplexes were analyzed by SDS-polyacrylamide gel (7.5%) electrophoresis in reducing conditions (lanes 1-8) or non-reducing conditions (lanes 9-16). Lanes 1, 9 and 2, 10 represent the analysis of the culture media and the extracts, respectively, of non-transfected cells, lanes 3, 11 and 4, 12 for the culture media and the extracts, respectively, of pCD4ITM10-transfected cells, lanes 5, 13 and 6, 14 for the culture media and the extracts, respectively, of pCD4ITM20 transfected cells, and lanes 7, 15 and 8, 16 for the culture media and the extracts of pCD4ITM30-transfected cells.

Figure 20 shows the analysis of the binding properties of CD4(178)CH and soluble CD4(372) which were secreted from the CV-1-transfected cells. Lanes 1-6 represent the analysis of soluble CD4, and lanes 7-12 represent the analysis of CD4(178)CH. The culture media were incubated with the following antibodies and ligands, and analyzed on each lane. Lanes 1 and 7 (no addition), 2 and 8 (OKT4), 3 and 9 (OKT4A), 4 and 10 (gp120), 5 and 11 [anti-human IgG (Fc)], 6 and 12 (protein A-agarose). solCD4 and 178H denote soluble CD4(372) and CD4(178)CH, respectively.

Figure 21 shows the analysis of the binding property of CD4(178)CH which was secreted from the RPMI8226-transfected cells. The culture media were incubated with the following antibodies and ligands, and

- 12 -

analyzed on each lane. Lane 1 (OKT4), 2 (OKT4A), 3 (gp120), 4 [anti-human IgG (Fc)], 5 (protein A-agarose), 6 (anti-human lambda chain). 178H and IgL denote CD4(178)H and Ig light chain (lambda type), respectively.

5 Figure 22 shows the analysis of the binding properties of CD4(178)CH and CD4(181)CL coexpressed in CV-1 cells. The culture media of pCD4ITM20G and pCD4ITM40G doubly-transfected cells were incubated with the following antibodies and ligands, and analyzed on
10 each lane. Lane 1 (no addition), 2 (OKT4), 3 (OKT4A), 4 (gp120), 5 [anti-human IgG (Fc)], 6 (protein A-agarose), 7 (anti-human kappa chain). 178H and 161L denote CD4(178)Ch and CD4(181)CL, respectively.

DETAILED DESCRIPTION OF THE INVENTION

15 The above and various other objects and advantages of the present invention are achieved by a chimeric gene which encodes a recombinant fusion protein having selective toxicity against specific virus-infected cells. The principal aspect of the present invention is
20 that a toxin, or a cytotoxic part thereof, could be genetically attached to a receptor protein (or a fragment thereof) so that the fusion product binds to cells infected with a virus, since all viruses depend on a cellular receptor for entry. CD4 is one such receptor
25 required by HIVs of different types. According to the present invention, such a chimeric gene encodes recombinant hybrid proteins comprising the HIV-gp120 binding regions of the human CD4 molecule and constant regions of the human immunoglobulin heavy and/or light chain molecules. Hence, this invention is demonstrated by a partial but essential CD4 linked fusion cytotoxic product.
30 The same principle can be applied for other viruses.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as
35 commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described

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- 14 -

(λ DE3) carrying pVC403 was used to express the fusion protein upon IPTG induction. The direction of transcription from the T7 promoter and for the B-lactamase gene is shown by solid arrows in Figure 1. The circled numbers are the amino acids of CD4 and the boxed numbers are the amino acids of PE. The boundaries of the CD4(178) sequence and the start of PE40 sequence are shown at the top.

Construction of pVC403:

Plasmid pVC4 which carries a full length PE gene attached to a T7 promoter was cut with NdeI and Asp718 and ligated to a 52 bp linker containing codons for the first 16 amino acids of mature CD4 and NdeI and Asp718 cohesive ends. This intermediate plasmid (pVC401) has 3 RsaI sites, one between the first 16 codons of mature CD4 and the remainder of PE gene. pVC401 was partially cut with RsaI, then with XhoI, and a 2.8 Kb fragment was isolated. Plasmic pCD4SPE40TM1 which carries a fusion gene between the first 178 amino acids of CD4 [CD4(178)] and PE40 under a T7 promoter was restricted with RsaI and XhoI, and a 1.3 Kb fragment was isolated. Construction of pCD4SPE40TM1 is described below. The 1.3 Kb fragment from pCD4SPE40TM1 was ligated to a 2.8 Kb fragment from pVC401 to produce plasmid pVC403. This plasmid has a Nde I site at the junction of the CD4(178) and PE40 genes that can be used to introduce various other PE genes.

Construction of pCD4SPE40TM1:

A 0.70-kb EcoRI-SalI fragment containing the amino-terminal two immunoglobulin-like domains of CD4 was excised from pCD4f and cloned into M13mpl8. The resulting recombinant phage, mpl8CD4TM1 was propagated in a dut⁻ ung⁻ strain and the single-stranded template DNA was annealed with a 33 mer oligonucleotide, TM21 containing an NdeI site (CATATG) encoding histidine and methionine residues just after a codon encoding an alanine residue, the 178th amino acid of CD4. After second strand syn-

- 15 -

thesis, the double-stranded DNA was transformed into a wild type strain, and a mutant clone mpl8CD4TM21 was selected by NdeI digestion.

To obtain a final expression plasmid for a fusion protein more easily, an intermediate plasmid, pCD4PE40TM1 was constructed as follows. A 1.23-kb fragment containing PE40 was excised from pVC8 by digesting it with EcoRI, filling in the cohesive end with DNA polymerase I Klenow fragment, and digesting it with XbaI. The fragment was ligated with a 5.21-kb fragment of pCD4LTM1 (an expression plasmid for 372 amino-acid CD4); the fragment was obtained by digesting the plasmid with SalI, filling in the cohesive end with DNA polymerase I Klenow fragment, and digesting with NheI, yielding pCD4PE40TM1. From this plasmid, a 5.72-kb NdeI (partial)-EcoRI fragment was excised and ligated with a 0.69-kb NdeI-EcoRI fragment of mpl8CD4TM21, yielding pCD4SPE40TM1. This plasmid is capable of expressing a 546 amino-acid fusion proteing consisting of the first 178 amino acids of DC4 at the amino terminus, followed by histidine and methionine residues derived from the NdeI site used for joining the two molecules, followed by the carboxy terminal PE40 sequence.

A deposit of the plasmid pVC 403 has been made at the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, 20852, U.S.A., on June 29, 1988 under the accession number 67739. The deposit shall be viably maintained, replacing if it becomes non-viable, for a period of 30 years from the date of the deposit, or for 5 years from the last date of request for a sample of the deposit, whichever is longer, and made available to the public without restriction in accordance with the provisions of the law. The Commissioner of Patents and Trademarks, upon request, shall have access to the deposit.

Purification and Characterization of CD4(178)-PE40:

BL21 (λ DE3) carrying plasmid pVC403 was grown

- 16 -

in LB medium at 37°C with ampicillin (100 ug/ml), induced at $OD_{550nm}^{0.6}$ with 1mM isopropyl D-thiogalactoside (IPTG) and the incubation continued for 90 minutes at 37°C. The cells were fractionated into periplasm and spheroplasts. The spheroplasts were suspended in TE (50 mM Tris pH 8.0, 1mM EDTA), sonicated three times at 100 watts for 30 seconds each and spun at 100,000 x g for 60 minutes to isolate the supernatant (cytoplasm) and pellet (containing inclusion bodies). For localization experiments, the pellet was suspended in TE and the various fractions were analyzed by ADP-ribosylation assays and by SDS-PAGE, using Coomassie Blue staining and immunoblotting with rabbit antibodies to PE.

For partial purification of CD4(178)-PE40, a denaturation/renaturation procedure was employed. A 500 ml culture of BL21 (λ DE3) containing pVC403 was induced and the inclusion body pellet fraction prepared as described above. The pellet was suspended in 6.5 ml extraction buffer (guanidine HCl 7M, Tris HCl 0.1M pH 8.0, EDTA 1mM and DTT 1mM) and sonicated for 20 seconds three times. The suspension was stirred for 1 hour in the cold and centrifuged at 100,000 x g for 15 minutes, and the supernatant saved. The supernatant (6.5 ml) was added dropwise to 500 ml cold phosphate buffered saline with rapid stirring. After 48 hours, a portion was purified as follows: 110 ml was dialyzed against Buffer A (Tris HCl 20 mM pH 7.7) for 8 hours with two one liter changes, filtered through a 0.45 μ m filter and applied on a Mono Q column (HR 5/5) at a flow rate of 1 ml/min. The column was washed with 5 ml buffer A and then developed with a 25 ml linear gradient (0-0.5 M NaCl) and finally with 5 ml 1 M NaCl in Buffer A. 1 ml fractions were collected and analyzed for total protein, ADP-ribosylation activity and reactivity by ELISA using immobilized anti-PE antibodies and anti-CD4 monoclonal antibodies OKT4A (Ortho) and BL41 (Pel-freeze). Protein concentration was determined using Bradford reagent with bovine

- 17 -

serum albumin as a standard. ADP-ribosylation activity is expressed as units/ml; 1 unit is equal to the activity of 1 μ g of PE40 determined under the same assay conditions. For SDS-PAGE, samples were boiled with Laemmli sample buffers and electrophoresed on 10-15% gradient gels (PhastGels, Pharmacia). Analysis of the purification is shown in Fig. 2:

5 (a) Mono Q column chromatography of renatured soluble CD4(178)-PE40: 110 ml of renatured material (6 mg protein) was applied to a Mono 1 column; proteins were eluted with a NaCl gradient and fractions of 1 ml were collected.

10 (b) SCS-PAGE of samples at various stages of purification: Gels were either stained with Commassie blue (lanes 1-3), or immunoblotted with polyclonal antibodies to PE (lanes 4-6). Lanes 1 and 4, spheroplasts; lanes 2 and 5, fraction 19 from Mono Q column; lanes 3 and 6, authentic PE < 100 ng. Molecular weight of protein standards are shown in Kd. The molecular weight authentic PE is 66 Kd.

20 Demonstration of Binding of CD4(178)-PE40 to HIV Envelope Protein gp120:

Two methods were employed to demonstrate this specific binding interaction. The first involved coprecipitation of radiolabeled gp120 along with CD4(178)-PE40 plus antibodies to PE. Media containing [³⁵S]-methionine-labeled gp120 was obtained as previously described using a vaccinia/bacteriophage T7 hybrid expression system. CV-1 cells were co-infected with two vaccinia virus recombinants: vPE6, encoding a secreted form of gp120 (HIV-1, IIIB isolate) under control of the bacteriophage T7 promoter, and vTF7-3, encoding the T7 RNA polymerase driven by the vaccinia virus 7.5 K promoter. Five μ l of media containing ³⁵S-labeled gp120 was preincubated with 90 μ l of crude renatured CD4(178)-PE40 for 4 hr at 4°C. Anti-PE antiserum (2 μ l) was added, and after overnight incubation at 4°C the immune complexes

- 18 -

were precipitated with 10 l Protein A-agarose (Calbiochem). The washed immunoprecipitates were analyzed by SDS-PAGE on 10% gels and the protein bands visualized by fluorography. The analysis is shown in Fig. 3A. The immune complex precipitated from a mixture of media containing [³⁵S]-labeled gp120 is shown in lane 3. For controls, either the anti-PE (lane 1) or the CD4(178)-PE40 (lane 2) were omitted.

The second method involved immunofluorescence. Confluent monolayers of CV-1 cells in 35 mm wells of 6-2311 plates (Costar) were infected with a recombinant vaccinia virus encoding gp160 (HIV-1, IIIB isolate) under control of the vaccinia 7.5K promoter. The multiplicity of infection was 1.5. As a control, cells were infected with vTF7-3 (see above). Ten hours post-infection, CD4(178)-PE40 (fraction 19 of the mono Q column, see Fig. 2) was added to a final toxin concentration of 50 µg/ml in PBS plus 0.2%-(w/v) bovine serum albumin. After 1 hr at 4°C, the dishes were rinsed and incubated with a polyclonal anti-PE antiserum [1:500 in PBS plus 0.2% (w/v) bovine serum albumin] for an additional hour at 4°C. The cells were then incubated with affinity purified goat anti-rabbit IgG conjugated to rhodamine. Cells were fixed in formaldehyde, mounted and photographed. In the analysis shown in Figures 3B, 3C, 3D and 3E cells were infected with either the recombinant vaccinia virus containing the HIV-1 gp160 gene (3B and 3C) or with the control vaccinia recombinant (3D and 3E). 3B and 3D are fluorescence micrographs and 3C and 3E are the corresponding phase contrast micrographs. Bar indicates 20 µm at 350x.

Demonstration of Selective Cytotoxicity for Cells Expressing the HIV Envelope Glycoprotein:

Two test systems were employed. The first involved cells expressing the HIV envelope glycoprotein encoded by a recombinant vaccinia virus. Duplicate essays were performed in 16 mm wells of 24-well plates

- 19 -

(Costar). CV-1 cells were grown to 90% confluence (2×10^5 cells per well). The indicated recombinant vaccinia viruses were added to the wells at a multiplicity of infection of 20 in 0.25 ml of Dulbecco's MEM supplemented with 2.5% fetal bovine serum. After 90 min with occasional rocking, the medium was removed and replaced with 1 ml of the same medium containing 10% of the normal methionine concentration. 7.5 hr later, 0.05 ml of the indicated toxin preparations in Dulbecco's phosphate buffered saline were added to give the final concentrations shown. The incubations were continued for 4 hr, at which time 20 μ Ci of ^{35}S -methionine was added to each well in 0.05 ml of methionine-free medium. After one hr the labeling medium was removed and the wells were rinsed twice with 1 ml of Dulbecco's phosphate buffered saline. Cells were harvested in 0.5 ml of 0.1 N NaOH containing 0.1% (w/v) bovine serum albumin and the protein was precipitated with trichloroacetic acid and radioactivity determined by scintillation counting.

Results are expressed as % control incorporation (no toxin added). In the analysis shown in Fig. 4a, closed symbols represent cells infected with a vaccinia recombinant encoding gp160, whereas open symbols represent cells infected with a control vaccinia recombinant, vTF7-3, encoding the bacteriophage T7 RNA polymerase (see description of Fig. 3). The toxin preparations used were (Δ , \blacktriangle) PE, (O, \bullet) PE40, (\square , \blacksquare) CD4(178)-PE40.

The second test system employed cells chronically infected with HIV. 8E5 is a human T-cell line which contains a single integrated copy of the HIV-1 (LAV) genome. The virions produced are non-infectious due to a premature chain termination mutation in the reverse transcriptase gene. As a control, the parental non-infected A3.01 cell line was used. Assays were performed in duplicate in 24-well plates. Individual wells were seeded with 7×10^5 cells of the indicated cell line in 0.9 ml of medium containing 1 part RPMI supplemented

- 20 -

with 10% fetal bovine serum plus 8 parts of the same medium lacking methionine. The designated toxin preparations were added in 0.02 ml Dulbecco's PBS to give 111% of the final concentration shown. After 17.5 hr, 10 μ Ci of 35 S-methionine in 0.1 ml of complete medium was added to each well, and the incubations continued for 1 hr.

Contents of the wells were then quantitatively transferred to centrifuge tubes and spun for 10 min at 2800 RPM in a Beckman centrifuge. The supernatants were removed and the pellets suspended in 0.1% (w/v) SDS. Fifty 1 aliquots were analyzed by trichloroacetic acid precipitation using Whatman GF/C filters. Results are expressed as % control incorporation (no toxin added). A background value was obtained by treating cells with 5 g PE for 18.5 hr, then incubating with 35 S-methionine for only 1 min. This value, which represented only 3% of the control incorporation, was subtracted in the calculations to yield the data shown in Fig. 4b. Closed symbols represent the 8E5 cells, whereas open symbols represent the A3.01 cells. The toxin preparations used were: (Δ , \blacktriangle) PE, (\circ , \bullet) PE40, (\square , \blacksquare) CD4(178)-PE40.

RESULTS

As shown in Figure 1, a chimeric gene encoding the first 178 amino acids of CD4 and amino acids 1 to 3 and 253 to 613 of PE was constructed (Figure 1). This segment of PE (designated PE40) lacks domain I but retains domains II and III which are responsible for translocation and ADP-ribosylation, respectively. The plasmid, pVC403, also contained a bacteriophage T7 late promoter and the Shine-Delgarno ribosome binding sequence for high expression in *Escherichia coli* BL21 (λ DE3). The chimeric protein, designated CD4(178)-PE40, was synthesized in large amounts, remained intracellular and appeared to be primarily associated with inclusion bodies in the 100,000xg pellet of sonicated spheroplasts. The denatured protein had the expected Mr of approximately 60,000 and reacted with polyclonal antibodies to PE by

- 21 -

immunoblot analysis (see below).

A purification scheme involving denaturation of the insoluble protein with guanidine followed by rapid dilution was used. An ELISA demonstrated that the renatured form of CD4(178)-PE40 reacted with polyclonal antibody to native PE and monoclonal antibodies OKT4A and BL4 directed to CD4. Enzymatic activity of the hybrid protein was shown by an affinity capture procedure; up to 30% of the ADP-ribosylation activity could be selectively immunoprecipitated by OKT4A monoclonal antibody.

A highly purified monomeric form of CD4(178)-PE40 was obtained by chromatography of the renatured protein on a mono Q column (Figure 2A). Analysis of each fraction by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) indicated that fraction 19 contained at least 80% pure monomeric fusion protein that reacted with a rabbit polyclonal antibody to PE (Figure 2). Further purification of fraction 19 on TSK-250 gel filtration column showed that the fusion protein eluted as a symmetrical peak at the elution volume expected for a 60,000 Mr protein. SDS polyacrylamide gel electrophoresis revealed the presence of a single band corresponding to a protein of about 60,000 Daltons.

To determine the ability of CD4(178)-PE40 to bind gp120, two types of assays were performed (Figure 3). First, CD4(178)-PE40 was mixed with soluble [³⁵S]methionine-labeled gp120, and the immune complexes obtained with anti-PE serum were bound to protein A agarose and resolved by SDS polyacrylamide gel electrophoresis. As shown in Figure 3A, labeled gp120 was specifically coprecipitated along with CD4(178)-PE40. Second, the binding of CD4(178)-PE40 to cell-associated gp120 was established by immunofluorescence microscopy. The HIV envelope glycoprotein was produced in CV-1 cells using a vaccinia based expression system. Both the external gp120 and transmembrane gp41 subunits are

- 22 -

present on the surface of cells infected with a recombinant vaccinia virus encoding gp 160; furthermore, such cells form extensive syncytia when mixed with CD4-bearing human cells. Figure 3B shows that CD4(178)-PE40 bound to CV-1 cells that had been infected with a recombinant vaccinia virus encoding gp160 but not to cells infected with a control recombinant vaccinia virus. Additional specificity controls indicated that the fluorescence was not observed if either the CD4(178)-PE40 or the anti-PE antibodies were omitted. Taken together, these results demonstrate that CD4(178)-PE40 binds to gp120 in solution or on the cell surface.

Binding of authentic PE to cells followed by internalization and translocation to the cytoplasm results in ADP-ribosylation of elongation factor 2 and the consequent inhibition of protein synthesis and cell death. Two assay systems were employed to determine whether CD4(178)-PE40 is selectively internalized and translocated by cells expressing the HIV envelope glycoprotein, leading to inhibition of protein synthesis. First, the effects on cells infected with recombinant vaccinia viruses were examined.

Figure 4A shows that protein synthesis in CV-1 cells infected with a recombinant vaccinia virus encoding gp160 was severely inhibited after only 4 hours of exposure to CD4(178)-PE40 (ID_{50} of 27 ng/ml). By contrast, cells infected with a control recombinant vaccinia virus were much less sensitive to the hybrid toxin ($ID_{50} > 1,000$ ng/ml). When authentic PE was employed, cells infected with the gp160-expressing and the control recombinant vaccinia viruses were equally sensitive ($ID_{50} = 100$ ng/ml); they were also equally insensitive to PE40, which lacks a cell binding domain ($ID_{50} > 1,000$ ng/ml). We conclude that expression of the HIV envelope protein rendered cells very sensitive to the hybrid toxin and that the specificity was conferred by the CD4 moiety.

As a second system to evaluate the selectivity

- 23 -

and effectiveness of CD4(178)-PE40, an uninfected human lymphocyte cell line (A3.01) and a daughter cell line (8E5) that is chronically infected with HIV were tested as targets. The 8E5 cells are especially suitable for experimental studies since they contain a single integrated viral genome, constitutively synthesize HIV proteins including gp120, form syncytia when mixed with CD4-bearing cells, and produce budding particles. Addition of CD4(178)-PE40 to 8E5 cells led to inhibition of protein synthesis: the ID₅₀ of 100 ng/ml (determined at 17.5 hours after addition of toxin) indicated that the HIV-infected cells were highly sensitive to the hybrid toxin (Figure 4B). By contrast, protein synthesis in the parental A3.01 cells was resistant to CD4(178)-PE40 under these conditions. Both cell lines were moderately sensitive to authentic PE (ID₅₀ = 500 ng/ml) and unaffected by PE40.

In evaluating the therapeutic potential of a hybrid toxin, effects on cells other than the desired targets must be considered. Since the natural receptor for CD4 is believed to be the class II major histocompatibility (MHC) molecules on the surface of antigen-presenting cells, B-lymphocytes and macrophages might be affected by the chimeric toxin. Tests indicated, however, that CD4(178)-PE40 did not inhibit protein synthesis in Raji cells, a B-cell line which expresses large amounts of MHC class II molecules. This result is consistent with a published report that soluble CD4 has no inhibitory effect on CD4/MHC class II interactions in vitro, and suggests that monomeric forms of CD4 may have relative weak affinity for class II antigens.

These results demonstrate that HIV-infected lymphocytes were selectively killed by a hybrid toxin made in *E. coli* containing a 178 amino acid segment of human CD4 linked to the second and third domains of the potent Pseudomonas exotoxin A. Additional experiments with recombinant vaccinia virus expression vectors indi-

- 24 -

cated that the sensitivity to the chimeric toxin resulted from expression of the HIV envelope protein. For both the HIV-infected and the recombinant vaccinia virus-infected cell systems, selectivity was mediated by the CD4 moiety of the chimeric protein. The concentrations of purified renatured CD4(178)-PE40 required for 50% inhibition of protein synthesis in three experiments ranged from 27 to 100 ng per ml. Based on data with other PE fusion proteins, it is not difficult to attain such levels in animals without significant non-specific toxicity. Furthermore, CD4(178)-PE40 could be useful against cells infected with diverse strains of HIV-1 as well as HIV-2, since the envelope proteins of all these viruses retain binding specificity for CD4 despite extensive antigenic variation.

In summary, the data presented herein clearly establish that a fusion protein CD4(178)-PE40 specifically and efficiently kills HIV-infected cells. This allows the use of this recombinant toxin as a therapeutic agent for the control and treatment of AIDS. A therapeutic composition in accordance with the present invention comprises an effective amount of the recombinant toxin to kill HIV-infected cells in a pharmaceutically acceptable vehicle, if necessary, such as physiological saline, buffered solutions and the like. The toxin may be administered by any suitable route, systemically or locally as deemed more effective. The method of controlling or treating AIDS comprises contacting HIV-infected cells with the effective amount of the recombinant toxin [CD4(178)-PE40 fusion protein] to kill HIV-infected cells or inhibit fusion and syncytia formation resulting subsequent to HIV-infection.

It is important to note here that the present invention differs significantly from and has advantages over other treatment modalities of AIDS at least in the following respects.

I. It has been reported that soluble deriva-

- 25 -

tives of CD4 block HIV infectivity of cells in culture, presumably by competing for the ability of the virus to bind to cell-associated CD4. Without being bound to any specific theory, it is postulated that the invention described herein acts by a different mechanism, namely by killing cells which have already been infected with HIV. In this regard, it has been reported that soluble CD4 is much less effective when added after the virus has been allowed to infect the cell, unlike the CD4-toxin which kills cells after infection has occurred. It should be noted that the hybrid toxin may also produce competitive inhibition of infectivity seen with soluble CD4 in addition to its targeted killing of HIV-infected cells.

II. Selective killing of HIV-infected cells using an immunotoxin composed of an anti-gp120 mouse monoclonal antibody chemically conjugated to protein toxin (ricin) has also been reported. However, the CD4(178)-PE40 fusion protein of the present invention possesses numerous advantages over this immunotoxin: (a) In the case of the immunotoxin the antibody used is type specific, and does not bind to gp120 from diverse isolates of HIV-1. In contrast, CD4(178)-PE40 may be used against divergent strains of HIV-1 as well as against HIV-2, since all these viruses use CD4 as the receptor. Because of this requirement for CD4 receptor specificity, it is extremely unlikely that variants of HIV, resistant to CD4-toxin hybrid proteins, will arise, whereas variants which no longer bind type-specific monoclonal antibodies often arise. (b) The immunotoxin is produced by chemical coupling procedures which are difficult to control, thereby compromising the uniformity of the conjugate and also result in low yield. In contrast, the recombinant CD4(178)-PE40 fusion protein can be produced in large quantities in a bacterial expression system using standard procedures. (c) The mouse immunoglobulin component of the immunotoxin is likely to be

- 26 -

immunogenic in human subjects, thereby compromising its effectiveness. In contrast, with CD4-toxin fusion proteins, the targeting of gp120-expressing cells is achieved by a fragment of human CD4, which is likely to be less immunogenic in humans.

III. Selective killing of HIV-infected cells in vitro by liposomes containing diphtheria toxin fragment A has also been reported. Clearly, this is quite distinct from the fusion-protein methodology of the present invention.

Having described certain aspects of the present invention, various modifications thereof which can be achieved by one of ordinary skill in the art, are now listed.

A. Variations in the CD4 portion. This can be achieved, for example, by differences in length of the CD4 sequencer. Shorter or longer versions of the CD4 sequence can be found which can also be attached to toxins to achieve selective killing of HIV-infected cells. The length of the CD4 sequence can have important consequences for the affinity for gp120, for the relative affinities for gp120 vs. class II antigens, for the physical accessibility to different regions within the body, and for the immunogenicity. In addition, site-specific mutagenesis can be used to decrease the affinity of CD4 for normal cellular antigens, and/or increase the affinity for gp120. Such mutations would widen the window between effective therapeutic dosages and unwanted toxic side effects.

B. Variation in the toxin portion. Modifications of PE can be made. By selective mutagenesis or deletion, the immunogenicity of the PE sequence can be reduced and the potency of the hybrid toxin increased (e.g., by enhancing translocation such as ricin and diphtheria toxin fragment A could be similarly employed in context of fusion technique described herein.

C. Expression systems

- 27 -

Bacterial. By employing, for example, certain E. coli expression system, secreted forms of the hybrid toxin can be made obviating the need for denaturation/re-naturation.

5 Eukaryotic. Mammalian, vaccinia virus, baculovirus, and yeast expression systems can also be used as advantageous expression systems as is well known to one of ordinary skill in the art.

PART II

MATERIALS AND METHODS

10 Enzymes. Restriction endonucleases were obtained from New England Biolabs or Bethesda Research Laboratories. The Klenow fragment of DNA polymerase I and T4 DNA ligase were from New England Biolabs.

15 Antibodies. Murine anti-CD4 mAbs were obtained from the following sources: MT151, Boehringer Mannheim; Leu3A, Becton Dickinson; OKT4, OKT4A, OKT4B, OKT4C, OKT4D, OKT4E, and OKT4F, M. Talle, Ortho Diagnostics. Two murine anti-gp120 mAbs were employed: 2E12.1
20 (Epitope, Beaverton, OR) and a tissue culture supernatant from hybridoma 902 (B. Chesebro, National Institute of Allergy and Infectious Diseases, Hamilton, MT). Rabbit antiserum to mouse IgG (heavy plus light chains) was purchased from ICN.

25 Plasmids. The CD4 cDNA was donated by D. Littman (University of California, San Francisco). Plasmid pCD4-GEM4 (obtained from A. Rabson, National Institute of Allergy and Infectious Diseases, Bethesda, MD) contains a full-length copy of the CD4 cDNA with
30 5' EcoRI and 3' BamHI linkers (Maddon et al, supra) cloned into the EcoRI-BamHI site of pGEM4 (Promega Biotec, Madison, WI). pTF7-5 contains a bacteriophage T7 promoter and terminator separated by a unique BamHI site and flanked by the left and right vaccinia thymidine kinase
35 gene sequences (see Fig. 1).

Construction and preparation of recombinant plasmids were performed according to the methods outlined

- 28 -

by Maniatis et al. 1982. Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Lab., Cold Spring Harbor, NY). DNA fragments were purified from low-melting-point agarose gels by using the Elutip-d procedure (Schleicher & Schuell). Plasmids were isolated by the alkaline NaDodSo₄ lysis method (Birnboim et al. 1979. Nucleic Acids Res. 7, 1513-1523) and purified by CsCl/ethidium bromide equilibrium density gradient centrifugation.

Virus and Cells. Vaccinia virus recombinant vTF7-3 contains the bacteriophage T7 gene 1 (encoding the T7 RNA polymerase) under control of the vaccinia P7.5 promoter (Fuerst et al. 1986. Proc. Natl. Acad. Sci. USA 83, 8122-8126). vPE6 is a vaccinia recombinant derived from pTF7-5 containing a bacteriophage T7 promoter linked to the HIV-1 envelope gene (IIIB isolate, clone BH8) with a termination codon inserted by in vitro mutagenesis immediately preceding the sequence encoding the consensus retroviral envelope cleavage site Arg-Glu-Lys-Arg. This virus directs high-level expression of a secreted form of gp120 in cells doubly infected with vTF7-3.

CV-1 monkey kidney cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

Expression Conditions. Transfection experiments for transient expression were performed by using conditions similar to those described in Fuerst et al, supra. CV-1 cells were grown to 90-95% confluence in 25-cm² flasks (2.5×10^6 cells) and infected with vTF7-3 at a multiplicity of 30 plaque-forming units (pfu) per cell in medium with 2.5% fetal bovine serum. The virus was allowed to adsorb for 30 min at 37°C with occasional rocking of the flask, whereupon the inoculum was removed and replaced with 1 ml of transfection buffer containing 5 ug of calcium phosphate-precipitated plasmid DNA. After incubation for 30 min at 37°C with occasional rocking, 5 ml of medium containing 2.5% fetal bovine serum

- 29 -

was added. The medium was removed after 4 hr at 37°C, and the cells were incubated with 2-4 ml of cysteine-free medium containing 2.5% dialyzed fetal bovine serum for 15-60 min. This medium was then replaced with 2.25 ml of
5 the same medium supplemented with 0.1-0.3 mCi of L-[³⁵S]cysteine per ml (Amersham, 1.3 Ci/mmol; 1 Ci = 37 GBq). Labeling was allowed to proceed at 37°C for 4-5 hr, after which 0.25 ml of complete medium containing 2.5% fetal bovine serum was added. Thirty-six hours
10 later the medium was collected and centrifuged in a Savant high-speed microcentrifuge, first at 2000 rpm for 5 min and then at 10,000 rpm for 30 min. The resulting supernatant was used for subsequent analyses.

For expression of secreted gp120, double virus
15 infections were performed by using protocols similar to those reported (Fuerst et al. (1987. Mol. Cell. Biol. 7, 2538-2544). CV-1 cells grown in flasks as described above were infected with vTF7-3 and vPE6 (15 pfu each per cell) in 1 ml of medium containing 2.5% fetal bovine
20 serum. After 90 min at 37°C, the virus inoculum was removed and replaced with fresh medium containing 2.5% fetal bovine serum. In the case of unlabeled infection, the incubation was continued at 37°C for 23 hr, after which the medium was collected. For metabolic labeling,
25 the incubation was continued for 10.5 hr, at which time the medium was removed and the cells were incubated for 15 min at room temperature in cysteine-free medium with 2.5% dialyzed fetal bovine serum. This medium was then replaced with 2.25 ml of the same medium supplemented
30 with 0.1 mCi of [³⁵S]cysteine per ml. After 5 hr of labeling at 37°C, 0.25 ml of complete medium containing 2.5% fetal bovine serum was added. The incubation was continued for an additional 7 hr and the medium was then collected. Media from these infections were centrifuged
35 as described above for the transfection experiments.

Control infections were performed identically, except that the virus inoculum contained vTF7-3 only (30

- 30 -

pfu per cell).

Radioimmunoprecipitation. The specific reaction conditions are similar to those noted in the description of the figures supra. Protease inhibitor buffer contained 0.1 mM N α -(p-tosyl)-lysine chloromethyl ketone, 0.1 mM L-1-tosylamido-2-phenylethyl chloromethyl ketone, 50 mM iodoacetamide, 0.01 mM leupeptin, and 70 Kallikrein units of aprotinin per ml in phosphate-buffered saline with 0.02% (wt/vol) sodium azide. Immune complexes collected with protein A-agarose (Calbiochem) were precipitated and washed three times by centrifugation in a Savant high-speed microcentrifuge at 3000 rpm for 5 min. NaDodSO₄/Polyacrylamide Gel Electrophoresis. The procedure of Laemmli, 1970, Nature (London) 227, 680-685, was employed, with the acrylamide concentrations specified in the figure legends. Gels were analyzed by fluorography using EN³HANCE (New England Nuclear). ¹⁴C-Methylated protein molecular weight markers (Amersham) were lysozyme (M_r 14,000), carbonic anhydrase (M_r 30,000), ovalbumin (M_r 46,000), bovine serum albumin (M_r 69,000), phosphorylase b (M_r 93,000), and myosin (M_r 200,000).

RESULTS

Expression of the Soluble CD4 Fragment. The expression system employed in the present study is based on that of Fuerst et al, supra. Mammalian cells are infected with a recombinant vaccinia virus (vTF7-3) containing the bacteriophage T7 RNA polymerase gene linked to a vaccinia promoter and then transfected with a plasmid vector containing the target gene of interest flanked by bacteriophage T7 promoter and transcriptional terminator regions. The T7 RNA polymerase mediates high-level transcription of the target gene in the cytoplasm of the transfected cells. In accordance with the present invention, a plasmid vector (pEB-2) was designed that contains, between the T7 promoter and transcriptional terminator, two new unique restriction sites (EcoRI and

- 31 -

Stu I) directly followed by a universal translational termination sequence. After cleaving this vector with EcoRI and StuI, any DNA fragment containing an EcoRI site and a 3' blunt end can be force-cloned in the proper orientation. If the DNA insert contains the translation initiation codon but only a portion of the adjacent coding region for a particular gene, a truncated polypeptide is expressed. Depending on which synthetic termination codon is in frame, the shortened polypeptide may also contain up to three additional C-terminal amino acids encoded by the vector.

For the present studies, the EcoRI-Nhe I DNA fragment of the CD4 cDNA was inserted into pEB-2 to obtain another plasmid, designated pCD4 (Fig. 5). Based on the reported cDNA sequence (Maddon et al, supra), pCD4 would be expected to encode a truncated variant of CD4 with a normal N terminus; cleavage of the signal sequence would result in a polypeptide containing the first 177 amino acid residues of mature CD4 (the first two immunoglobulin-like domains) and containing no consensus N-linked glycosylation sites. This fragment might therefore be expected to be secreted into the medium. DNA sequence analysis of pCD4_f indicated that the fragment also contains C-terminal proline and arginine residues derived from the UTS sequence of the vector.

Figures 6A and 6B show the results of a transient metabolic labeling experiment with cells infected with vTF7-3 and transfected with different plasmids. NaDodSO₄/polyacrylamide gel electrophoretic analysis revealed that the medium of cells transfected with control plasmid pEB-2 contained a complex pattern of polypeptide bands (lane 3); medium from cells transfected with pCD4_f contained the same complex pattern of polypeptides as well as an additional faint band at the position expected for the truncated CD4 polypeptide encoded by this plasmid (lane 1). Immunoprecipitation analysis with a mixture of several murine anti-CD4 mAbs confirmed

- 32 -

that this band indeed represented a fragment of the CD4 molecule. It was selectively removed from the medium after immunoprecipitation (lane 2) and was specifically precipitated from the medium of cells transfected with pCD4_f (lane 5) but not pEB-2 (lane 6). The amounts of this labeled polypeptide that could be immunoprecipitated from the medium fraction far exceeded the amounts precipitable from the detergent-solubilized cell pellet fraction (data not shown). It was, therefore, concluded that pCD4_f encodes the expected fragment representing the N-terminal 177 amino acid residues of the extracellular region of the CD4 molecule (plus two residues from the vector), that this fragment is secreted in soluble form into the medium, and that it displays reactivity with anti-CD4 mAbs.

Epitope Analysis. By using a variety of assays, several laboratories have demonstrated that specific anti-CD4 mAbs differ widely in their ability to block the interaction between CD4 and the HIV envelope glycoprotein (McDougal et al, supra; Sodroski et al, supra; Lasky et al. 1987. Cell 50, 975-985; Ludin et al, supra; McDougal et al. 1986. J. Immunol. 137, 2937-2944; Sattenatu et al, supra). It was therefore of interest to test which epitopes are expressed on the soluble CD4 fragment encoded by pCD4_f. Fig. 7 shows the results obtained by radioimmunoprecipitation analysis from the medium of cells metabolically labeled after transfection with pCD4_f. Importantly, a close correlation was found between the reactivity of a particular mAb with the fragment and the ability of the mAb to block the CD4-envelope glycoprotein interaction. Thus, strong immunoprecipitation was obtained with mAbs MT151, Leu3A, OKT4A, OKT4B, OKT4D, OKT4E, and OKT4F, all of which have been shown to inhibit the interaction of membrane-associated or soluble gp120 with CD4. By contrast, mAb OKT4 failed to immunoprecipitate the fragment, consistent with its reported inability to block CD4 interaction with membrane-

- 33 -

associated or soluble gp120. mAb OKT4C displayed barely detectable reactivity with the fragment in keeping with its reported failure to block interaction of intact HIV with CD4 (McDougal et al, supra; Sattenatu et al, supra) and its relatively weak capacity to inhibit binding of soluble gp120 to CD4 (Lundin et al, supra).

Several of the anti-CD4 mAbs were also tested in an alternative assay that measures the ability of unlabeled medium from transfected cells to block the binding of a particular antibody to CD4 on the surface of a lymphocyte cell line. The results supported the immunoprecipitation analysis: medium from cells transfected with pCD4_f blocked the binding of mAbs MT151, Leu3A, and OKT4A but had no effect on the binding of mAb OKT4 (data not shown). It was therefore, concluded that the epitopes detected by the HIV-blocking anti-CD4 mAbs are contained within the N-terminal 177 amino acid residues of the extracellular region of CD4 and that functional epitopes for these mAbs can be produced when less than half of the full-length CD4 molecule is synthesized.

Interaction of the CD4 Fragment with Soluble gp120. It was next determined whether the CD4 fragment is capable of specific interaction with gp120. To test for the formation of a complex between these molecules, medium from metabolically labeled cells expressing soluble gp120 was mixed with medium from metabolically labeled cells expressing the CD4 fragment. It was then assessed whether a murine anti-gp120 mAb could specifically coprecipitate the CD4 fragment along with the gp120. The results of such a coprecipitation experiment are shown in Fig. 8A. Of the many labeled bands observed in the initial mixture of media containing gp120 and the CD4 fragment (lane 1), the anti-gp120 mAb specifically precipitated two proteins: gp120 and the CD4 fragment (lane 6). The identity of the CD4 fragment band was confirmed by its absence when the fragment-containing

- 34 -

medium was omitted from the reaction (only the gp120 band was observed) (lane 3) and by its comigration with the single band observed when the fragment-containing medium was immunoprecipitated with an anti-CD4 mAb (lane 7).

5 The presence of the CD4 fragment in the anti-gp120 immunoprecipitate resulted from a true complex with gp120, as judged by the absence of the fragment band when either normal medium (lane 4) or medium from metabolically labeled cells infected only with the vaccinia
10 virus expressing T7 RNA polymerase (lane 5) was used in place of the gp120-containing medium. Analysis of the supernatants remaining after immunoprecipitation indicated that under the conditions of this experiment, nearly all of the CD4 fragment was complexed to gp120,
15 and immunoprecipitation of the complex by the anti-gp120 mAb was virtually complete (data not shown). This indicated a high affinity for the binding reaction.

To further analyze the specificity of this interaction, it was further determined whether soluble
20 unlabeled gp120 could compete for the immunoprecipitation of the CD4 fragment by an anti-CD4 mAb. As shown in Fig. 8B, unlabeled medium containing gp120 strongly inhibited the immunoprecipitation of the CD4 fragment by the OKT4A mAb (compare lanes 1 and 3). By contrast, medium lacking
25 gp120 (from cells infected only with the vaccinia expressing T7 RNA polymerase) had no effect (lane 2). It was, therefore, concluded that the interaction of the CD4 fragment with gp120 is quite specific.

In summary, the results presented here provide
30 direct evidence for localization of the HIV-binding site within the N-terminal 177 amino acid residues of CD4, which contain the first two immunoglobulin-like domains.

The present invention now makes it possible to prepare a composition for the prevention of HIV infection. Such a composition comprises an effective amount
35 of the recombinant, soluble, truncated form of the CD4 containing the first two immunoglobulin-type domains

- 35 -

within the first 177 amino acid residues from the N-terminal half of the CD4 molecule, to inhibit binding of the HIV to the host cells. A method for inhibiting HIV infection of the host cells comprises providing an effective amount of the recombinant, soluble truncated form of CD4 molecule of the present invention, to bind HIV.

PART III

Without being bound to any specific theory or postulate, the following unique aspects of the third part of the present invention relating to CD4-immunoglobulin hybrid proteins are set forth.

(a) The invention relies on basic host immunological defense mechanisms which normally involve humoral antibody. By substituting the gp120 binding region of CD4 for the variable regions of immunoglobulins, the resultant molecules acquire high affinity for all HIV envelope variants, plus selected effector function provided by the particular heavy chain constant regions employed. This overcomes the problems associated with the failure of HIV-infected individuals to raise high affinity antibodies against conserved determinants of the HIV envelope glycoprotein.

(b) The hybrid molecules of the present invention contain only human sequences, thereby minimizing problems arising from host immune responses to foreign proteins.

(c) The present invention does not involve the use of molecules which are inherently cytotoxic. Thus, problems related to non-specific toxicity are greatly reduced compared to therapies involving CD4-toxins and immunotoxins.

(d) The CD4-immunoglobulin hybrid proteins of the present invention are disulfide-bonded multimers (dimers or tetramers, depending on the particular constructs). Multivalency greatly enhances the avidity of CD4 for gp120 on both the cell surface and the virion, compared to the monomeric CD4 derivatives. This also

- 36 -

enhances direct neutralization of viral infectivity or inhibition of viral spread by cell-to-cell fusion.

(e) The immunoglobulin region also prolongs the survival of the CD4 derivatives in the circulation, thereby enhancing beneficial effects mediated by both direct neutralization and effector-mediated mechanisms.

(f) CD4-immunoglobulin hybrid proteins may lyse intact virions as well as HIV-infected cells, in contrast to CD4 toxins which act only against cells. Indeed, there is support for complement-mediated lysis of certain retroviruses in vitro (Cooper et al, 1979. Springer Semin. Immunopathol, 2, 285-320).

In order to produce the CD4-immunoglobulin hybrid proteins of the present invention (shown schematically in Fig. 9), a number of factors must be considered. The first involves the region of CD4 to be employed. Most critically, it must contain the high affinity binding site for gp120. Experiments with truncated soluble derivatives of CD4 (Traunecker et al. 1988, Nature (London) 331, 84-86; Berger et al, supra) indicate that the amino terminal half of the extracellular region (approximately 180 amino acid residues, representing the first two immunoglobulin-like domains) contains the gp120 binding site. Site directed mutagenesis studies [Clayton et al. 1988, Nature (London) 335, 363-366; Landau et al. 1988, Nature (London) 334, 159-162; Peterson et al. 1988, Cell 54, 65-72; Mizukami et al, supra] suggest that the amino-terminal immunoglobulin-like domain (approximately the first 100 amino acids) is of particular importance and may be sufficient for gp120 binding.

Other factors to consider in choosing the CD4 sequence include the ease of expression of the corresponding immunoglobulin hybrid proteins in secreted form, their stability in the circulation, their accessibility to different sites in the body, and the possibility that they may also contain the determinants for binding MHC Class II antigens (the surface molecules on antigen-

- 37 -

presenting cells with which cellular CD4 is believed to interact). Such binding could potentially impair the activity of CD4- cells in normal function, though experiments with soluble CD4 [Hussey et al. 1988, Nature (London) 331, 78-81] and with CD4-Pseudomonas exotoxin hybrid protein (Berger et al, submitted) suggest that these effects will be negligible due to the weak binding affinity between soluble CD4 and MHC Class II molecules.

5 A second consideration is the choice of human immunoglobulin sequences. An effective approach is to link active regions of CD4 to the constant regions of immunoglobulin heavy chain. The resultant molecules contain the regions involved in binding to complement as well as to Fc receptors on the surface of cells participating in antibody-dependent cell mediated cytotoxicity (ADCC). As to immunoglobulins, preferred is human IgG1, since this immunoglobulin subclass has been shown to be the most efficient at mediating cell killing by both complement and ADCC (Bruggemann et al 1987, J. Exp. Med. 166, 1351-1361). This IgG segment contains the CH1, hinge, CH2 and CH3 regions. The resultant hybrid proteins are then secreted as disulfide-bonded homodimers which specifically bind gp120, antibodies to CD4 and to human IgG, Protein A, complement (specifically C₃), and Fc receptors on appropriate cells of the immune system (e.g., macrophages).

20 A third factor to consider is co-expression of the CD4-IgG heavy chain proteins along with human light chains. Under these conditions, disulfide-bonded heterotetramers analogous to normal human IgG are produced thereby enhancing expression and secretion of the CD4-heavy chain hybrid molecules. Furthermore, the binding of complement seems dependent on factors other than simply the presence of relevant sequences on the heavy chain constant region, since different subclasses of human IgG which contain the known complement binding sequence differ widely in their complement binding

- 38 -

capacity. Thus, higher order structural features appear to be important, and it is likely that these are dependent on the heterotetrameric structure with the light chains.

5 A particular embodiment of this approach involves co-expression of the CD4-heavy chain molecules, not with normal human light chains but instead with recombinant proteins containing CD4 sequences linked to the constant regions of human light chains. The resulting heterotetramers each contain four copies of the CD4
10 sequence, and this multivalency leads to extremely high avidity for gp120 on the surface of infected cells and HIV virions. The construction of the chimeric genes and the expression of the hybrid proteins are now exemplified.
15

MATERIALS AND METHODS

Materials

Restriction enzymes were purchased from New England BioLabs or Bethesda Research Laboratories. T4
20 DNA ligase was from New England BioLabs.

The following antibodies were obtained from the indicated sources: OKT4 and OKT4A (Ortho Pharmaceuticals); anti-Leu-3A (Becton Dickinson); a murine anti-human kappa light chain mAb (Boehringer Mannheim); a
25 murine anti-gp120 monoclonal antibody (mAb) from hybridoma 902 (National Institute of Allergy and Infectious Diseases, Hamilton, MT); biotinylated goat anti-mouse IgG and biotinylated goat anti-human IgG (Fc) antibodies (Bethesda Research Laboratories); biotinylated
30 goat anti-human lambda light chain antibody (Amersham).

Protein A-agarose and streptavidin agarose were purchased from Bethesda Research Laboratories.

Construction of the Intermediate Plasmid,

pCD4CH1 For easy construction of plasmids for expression of the hybrid proteins, the intermediate plasmid, pCD4CH1 was constructed as follows (Figure 10). A 1.7-kilobase (kb) EcoRI-BamHI fragment containing a whole
35

- 39 -

human CD4 cDNA was isolated from pDE4GEM4 (obtained from National Institute of Allergy and Infectious Diseases, Bethesda, MD). A 1.2-kb BamHI-SmaI(partial) fragment containing a partial human immunoglobulin G1 (IgG1) heavy chain cDNA including sequences coding for a joining (J) region and three constant domains (CH1, CH2, and CH3) was isolated from pGMH6 (Liu et al. 1987. Gene 54, 33-40). These two fragments were cloned into the StuI and EcoRI sites of pEB2, yielding pCD4CH1.

Construction of pCD4ITM10 and pCD4ITM10G To express the hybrid protein, CD4(109)CH (Figure 9) which comprises the first immunoglobulin (Ig)-like domain (1-109) of CD4 and three C domains of the human IgG1 heavy chain molecule, pCD4ITM10 and pCD4ITM10G were constructed as follows (Figure 11). A 0.46-kb EcoRI-NruI fragment encoding the amino-terminal 104 amino acids of CD4 was isolated from pCM34 in which an NruI site (TCGCGA) was inserted between the 103th and 104th codons of the CD4 cDNA (Mizukami et al, supra). The fragment, together with an adapter consisting of two synthetic oligonucleotides.

TM 44 (GACACCCACCTGCTTGCCTCCACCAAGGGCC) and

TM 45 (CTTGGTGGAGGCAAGCAGGTGGGTGTC).

was ligated into the ApaI and EcoRI sites of pCD4CH1 in which the ApaI site exists in the amino-terminal region of the CH1 domain. The resulting plasmid, pCD4ITM10 is capable of expressing CD4(109)CH under control of the bacteriophage T7 promoter. pCD4ITM10G was constructed by ligating a 2.2-kb XbaI-SalI fragment containing the coding sequences for CD4(109)CH from pCD4ITM10 with a 6.1-kb XbaI-SalI fragment from pTM3 which contains an Eco-gpt transcription unit as a selective marker.

Construction of pCD4ITM20 and pCD4ITM20G To express the hybrid protein, CD4(178)CH (Figure 9) which comprises the amino-terminal two Ig-like domains (1-178) of CD4 and three constant domains of the human IgG1 heavy chain molecule, pCD4ITM20 and pCD4ITM20G were constructed

- 40 -

as follows (Figure 12). An adapter consisting of two synthetic oligonucleotides, TM46 (CTAGCCGCCTCCACCAAGGGCC), and TM47 (CTTGGTGGAGGCGG), was ligated into the ApaI and NheI sites of pCD4CH1 in which the NheI site exists in the carboxyl-terminal region of the second domain of CD4. The resulting plasmid, pCD4ITM20 is capable of expressing CD4(178)CH under control of the bacteriophage T7 promoter, pCD4ITM20G was constructed by ligating a 2.4-kb XbaI-SalI fragment containing the coding sequences for CD4(178)CH from pCD4ITM20 with a 6.1-kb XbaI-SalI fragment from pTM3 which contains an Eco-gpt transcription unit as a selective marker.

Construction of pCD4ITM30 and pCD4ITM30G To express the hybrid protein, CD4(372)CH (Figure 9) which consists of the amino-terminal four domains (1-372) of CD4 and three constant domains of the human IgG1 heavy chain molecule, pCD4ITM30 and pCD4ITM30G were constructed as follows (Figure 13). A 0.65-kb SacI-HpaII fragment corresponding to nucleotides 598-1252 of the cDNA sequence reported by Maddon et al (1985, Cell 42, 93-104) was isolated from pCD4GEM4. The fragment, together with an adapter consisting of TM48 (CGGTGCAGCCAATGGCCTCCACCAAGGGCC) and TM49 (CTTGGTGGAGGCCATTGGCTGCAC), was ligated into the ApaI and SacI sites of pCD4CH1 in which the SacI site exists in the carboxyl-terminal region of the second domain of CD4. The resulting plasmid, pCD4ITM30 is capable of expressing CD4(372)CH under control of the bacteriophage T7 promoter. pCD4ITM30G was constructed by ligating a 3.0-kb XbaI-SalI fragment containing the coding sequences for CD4(372)CH from pCD4ITM30 with a 6.1-kb XbaI-SalI fragment from pTM3 which contains an Eco-gpt transcription unit as a selective marker.

Construction of pCD4ITM40G To express the hybrid protein, CD4(181)CL (Figure 9) which comprises the amino-terminal 181 amino acids of CD4, one amino acid (Leu) artificially created by introduction of a HindIII restriction site, three amino acids (Gln-Met-Lys) of the

- 41 -

joining region of the human Ig kappa light chain, and the whole constant region of the human Ig kappa light chain, pCD4ITM40G was constructed as follows (Figure 14). A 0.35-kb HindIII-XhoI fragment encoding a part of the joining region and the whole constant region of the human Ig kappa light chain was isolated from pING1480 in which the HindIII site was introduced into the joining region and the XhoI site was introduced into the 3 noncoding region of the human Ig kappa light chain cDNA. The fragment, together with an adapter comprising two synthetic oligonucleotides, TM62 (CTAGCTTTCAG) and TM63 (AGCTTCTGGAAAG), was ligated into the NheI and SalI sites of pCD4LTM1G, a derivative of pCD4LTM1 (Mizukami et al, supra), which contains an Eco-gpt transcription unit as a selective marker.

Expression of the hybrid proteins in CV-1 and RPMI8226 cells. Transient expression and metabolic labeling of the hybrid proteins with [35S] cysteine (Amersham) were performed according to the protocol of Fuerst et al (1986. Proc. Natl. Acad. Sci. USA 83, 8122-8126) by transfecting the expression plasmids for the hybrid proteins into the cells that were previously infected with vTF7-3 which expresses the bacteriophage T7 RNA polymerase gene under control of the vaccinia P7.5 promoter (Fuerst et al, supra). For subsequent analysis, 2.5 ml of culture medium was supplemented with 0.2 ml of 10-fold concentrated protease inhibitor buffer (Berger et al, supra) and 0.142 ml of 20% (vol/vol) Nonidet P-40. The cells were suspended with 1.25 ml of phosphate-buffered saline with 0.1 ml of 10-fold concentrated protease inhibitor buffer, and lysed by the addition of 0.071 ml of 20% (vol/vol) Nonidet P-40.

0.23 ml of the processed culture media or 0.115 ml of the cell extracts were added with 0.5 to 1.0 microgram of OKT4A and anti-Leu-3A, respectively, and incubated for 8 hr at 4°C. The immune complexes were collected by adding 0.1 ml of 20% (vol/vol) suspension of

- 42 -

protein A-agarose and washed as described by Berger et al (supra). The complexes bound to protein A-agarose were solubilized by boiling in Laemmli sample buffer containing 8 M urea and were resolved in SDS-polyacrylamide gels (Berger et al. supra). The gels were treated with Enhance (New England Nuclear) and labeled proteins were visualized by fluorography.

Conditions for binding of various antibodies and ligands to the hybrid proteins.

0.115 ml of the processed culture media were used for the binding experiments. For the binding to mAbs OKT4, OKT4A and mouse anti-human Ig kappa chain, the media were added with 0.5 microgram of the mAbs, incubated for 8 hr at 4°C, then added with 10 microgram of biotinylated goat anti-mouse IgG, and incubated for 8 hr at 4°C. 0.1 ml of 20% (vol/vol) suspension of streptavidin agarose was added, and the incubations were continued for 1 hr at 4°C on a rotator. The samples were processed and analyzed by SDS-polyacrylamide gel (7.5%) electrophoresis in a reducing condition as described by Berger et al (supra). For the binding to goat anti-human IgG (Fc) antibody and to goat anti-human Ig lambda light chain antibody, the media were mixed with 10 microgram of these biotinylated goat antibodies, and incubated for 8 hr at 4°C. 0.1 ml of 20% suspension of streptavidin agarose was added, and the samples were processed as above. For the binding to the HIV-1 gp120, the media were mixed with 0.1 ml of the culture media in which the gp120 had been expressed by a vaccinia virus-based double infection system (Fuerst et al, 1987, Mol. Cell. Biol. 7, 2538-2544), and incubated for 2 hr at 4°C. The complex was added with 0.1 ml of hybridoma 902 supernatant, incubated for 8 hr at 4°C, then added with 10 microgram of biotinylated goat anti-mouse IgG, and incubated for 8 hr at 4°C. 0.1 ml of 20% suspension of streptavidin agarose was added, and the samples were processed as above. For the binding to protein A-agarose, the media were directly added to 20%

- 42 -

protein A-agarose and washed as described by Berger et al (supra). The complexes bound to protein A-agarose were solubilized by boiling in Laemmli sample buffer containing 8 M urea and were resolved in SDS-polyacrylamide gels (Berger et al. supra). The gels were treated with Enhance (New England Nuclear) and labeled proteins were visualized by fluorography.

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- 43 -

(vol/vol) suspension of protein A-agarose, and the incubations were continued for 1 hr at 4°C on a rotator. The samples were then washed and processed as described above.

5

RESULTS

Three types of hybrid proteins [CD4(109)CH, CD4(178)CH and CD4(372)CH] with different lengths (1-109, 1-178, and 1-372 of the human CD4 extracellular region linked to human IgG1 heavy chain constant region, and one
10 hybrid protein [CD4(181)CL] comprising the amino-terminal 181 amino acid residues of CD4 and the human Ig light chain constant region were designed (Figure 9). To express these hybrid proteins, a vaccinia virus-based expression system was used, and the plasmids for the
15 expression of these hybrid proteins were constructed (Figures 10-14). In those plasmids the coding sequences for the hybrid proteins are placed under the T7 promoter and can be expressed upon coexpression of T7 RNA polymerase.

20 First, the expression of CD4(109)CH, CD4(178)CH, and CD4(372)CH in CV-1 cells was investigated (Figure 15). In both culture media and cell fractions, 38-kilodalton (kd), 65-kd, and 88-kd proteins were detected by transfection of pCD4ITM10, pCD4ITM20, and
25 pCD4ITM30, respectively, after immunoprecipitation with anti-CD4 mAbs followed by trapping with protein A-agarose. The calculated total amino acid residue numbers of CD4(109)CH, CD4(178)CH, and CD4(372)CH are 439, 508, and 702, respectively. The observed molecular weights of
30 the expressed proteins are slightly heavier than those estimated by the residue numbers. The differences presumably come from glycosylation of the proteins; the CH2 domain of the Ig heavy chain and the third and fourth Ig-like domains of CD4 each have one asparagine-linked
35 glycosylation site. A considerable portion of the synthesized proteins were secreted into the culture media even in the absence of Ig light chain expression; this

- 44 -

was unexpected, since it has been shown that Ig heavy chain secretion is poor in the absence of light chain expression (Pepe et al, 1986, J. Immunol. 137:2367-2372).

5 The subunit structure of the hybrid proteins which were expressed in CV-1 cells was then analyzed by SDS-polyacrylamide-gel electrophoresis in non-reducing conditions (Figure 16). In the absence of reducing agent, CD4(109)CH, CD4(178)CH, and CD4(372)CH moved to the high molecular weight positions, presumably in dimer
10 positions; by contrast a soluble form of CD4 consisting of 1-372 amino acid residues of CD4 migrated at the expected monomer position. The result indicates that CD4(109)CH, CD4(178)CH, and CD4(372)CH exist as disulphide-linked dimers both in the culture media and
15 inside of the cells.

Next, the expression of the other hybrid protein, CD4(181)CL, was examined by transfection of CV-1 cells with pCD4ITM40G (Figure 17). By transfection with only pCD4ITM40G, CV-1 cells expressed a 34-38 kd protein
20 which is most likely CD4(181)CL. The major portion of this protein migrated in the monomer position also in the absence of reducing agent, indicating that CD4(181)CL exists as monomers. In contrast, CD4(178)CH migrated in the dimer position in a non-reducing condition, indicat-
25 ing that CD4(178)CL exists as disulphide-linked dimers. When both proteins were coexpressed by cotransfection with pCD4ITM20G and pCD4ITM40G, a new protein band with a high molecular weight (>200 kd) appeared in a non-reduc-
30 ing condition. This band is most likely due to a hetero-tetrameric structure made of two subunits of CD4(178)CH and two subunits of CD4(181)CL, analogous to natural Ig molecules, and thus containing four HIV-gp120 binding sites (Figure 18).

35 Then, the coexpression of CD4(109)CH, CD4(178)CH, and CD4(372)CH with the normal human Ig lambda light chain was examined, by transfecting pCD4ITM10, pCD4ITM20, and pCD4ITM30 into RPMI 8226, a

- 45 -

human myeloma cell line (ATCC CCL 155) which secretes human Ig lambda light chains (Figure 19). All of those three hybrid proteins were synthesized and secreted efficiently into the culture media, although much of the expressed proteins accumulated in the inside of the cells. Co-immunoprecipitation of the human Ig lambda chain with CD4(109)CH, CD4(178)CH, and CD4(372)CH was observed, indicating that those hybrid proteins formed complexes with the human Ig lambda light chain which was synthesized by the host cell line. When the protein complexes were analyzed by SDS-polyacrylamide gels in a non-reducing condition, new protein bands with high molecular weights appeared for each complex. The estimated molecular weights of major complexes in the culture media of the cells transfected with pCD4ITM10, pCD4ITM20, and pCD4ITM30 are 180 kd, 210 kd, and >220 kd, respectively. This result indicates that these complexes represent tetrameric structures composed of two subunits of each hybrid protein and two subunits of the human Ig lambda light chain from the host cell. These molecules may, therefore, have structures analogous to natural Ig molecules.

The binding properties of CD4(178)CH to various ligands and antibodies was then investigated. CD4(178)CH which was expressed and secreted from the CV-1-transfected cells, was first analyzed (Figure 20). A soluble form of CD4 containing full extracellular four Ig-like domains was also expressed and analyzed as a control. CD4(178)CH bound to the HIV gp120, OKT4A, anti-human IgG (Fc) antibody, and protein A-agarose, but did not bind to OKT4. The soluble CD4 bound to the HIV gp120, OKT4, and OKT4A. However, it did not bind to anti-human IgG (Fc) antibody and protein A-agarose. The binding regions for gp120 and OKT4A have been identified in the first domain of CD4 (Clayton et al, supra; Landau et al, supra; Peterson et al, supra; Mizukami et al, supra), whereas the binding region for OKT4 is believed

- 46 -

to exist in the third or fourth domains of CD4. These results are consistent with the binding properties of CD4(178)CH and the soluble CD4 to the ligands and antibodies examined.

5 CD4(178)CH which was coexpressed with the host-derived human Ig lambda chain by the transfected-RPMI8226 cells was then examined for the binding property to various ligands and antibodies (Figure 21). The hybrid protein bound to the gp120, OKT4A, anti-human IgG (Fc) antibody, and protein A-agarose, but did not bind to 10 OKT4. When the Ig lambda chain was immunoprecipitated with anti-human Ig lambda chain antibody, CD4(178)CH was also coimmunoprecipitated with it, demonstrating that CD4(178)CH makes a complex with the host-derived lambda 15 chain.

CD4(178)CH and CD4(181)CL which were coexpressed in CV-1 cells were also analyzed for the binding properties to various ligands and antibodies (Figure 22). Although OKT4 did not immunoprecipitate CD4(178)CH or CD4(181)CL, OKT4A could immunoprecipitate both these 20 hybrid proteins. The gp120 could also bind to both proteins as well. Anti-human IgG (Fc) antibody and protein A-agarose could immunoprecipitate CD4(178)CH, and also coprecipitate CD4(181)CL with it. Anti-human Ig kappa 25 mAb could immunoprecipitate CD4(181)CL, and also coprecipitate CD4(178)CH with it. These results, together with the results shown in Figure 17, demonstrate that CD4(178)CH and CD4(181)CL coexpressed in CV-1 cells form the protein complex with a hetero-tetrameric structure in 30 which each subunit maintains its intrinsic binding property to various ligands and antibodies.

In summary, this part of the present invention teaches the construction of multimeric recombinant proteins comprising the gp120 binding region of human CD4 35 linked to constant regions of heavy and light chain components of human IgG, the resulting recombinant proteins at least possessing the property of inhibiting HIV

- 46 -

to exist in the third or fourth domains of CD4. These results are consistent with the binding properties of CD4(178)CH and the soluble CD4 to the ligands and antibodies examined.

5 CD4(178)CH which was coexpressed with the host-derived human Ig lambda chain by the transfected-RPMI8226 cells was then examined for the binding property to various ligands and antibodies (Figure 21). The hybrid protein bound to the gp120, OKT4A, anti-human IgG (Fc) antibody, and protein A-agarose, but did not bind to 10 OKT4. When the Ig lambda chain was immunoprecipitated with anti-human Ig lambda chain antibody, CD4(178)CH was also coimmunoprecipitated with it, demonstrating that CD4(178)CH makes a complex with the host-derived lambda 15 chain.

CD4(178)CH and CD4(181)CL which were coexpressed in CV-1 cells were also analyzed for the binding properties to various ligands and antibodies (Figure 22). Although OKT4 did not immunoprecipitate CD4(178)CH or CD4(181)CL, OKT4A could immunoprecipitate both these 20 hybrid proteins. The gp120 could also bind to both proteins as well. Anti-human IgG (Fc) antibody and protein A-agarose could immunoprecipitate CD4(178)CH, and also coprecipitate CD4(181)CL with it. Anti-human Ig kappa 25 mAb could immunoprecipitate CD4(181)CL, and also coprecipitate CD4(178)CH with it. These results, together with the results shown in Figure 17, demonstrate that CD4(178)CH and CD4(181)CL coexpressed in CV-1 cells form the protein complex with a hetero-tetrameric structure in 30 which each subunit maintains its intrinsic binding property to various ligands and antibodies.

In summary, this part of the present invention teaches the construction of multimeric recombinant proteins comprising the gp120 binding region of human CD4 35 linked to constant regions of heavy and light chain components of human IgG, the resulting recombinant proteins at least possessing the property of inhibiting HIV

- 47 -

proliferation by either neutralizing HIV activity, by killing HIV-infected cells, or by lysing HIV virions.

The availability of the unique recombinant proteins of the present invention now allows the preparation of a therapeutic or prophylactic composition comprising effective amount of the recombinant hybrid protein of the present invention to kill HIV or inhibit HIV infection, and pharmaceutically acceptable carrier. A method of treating or inhibiting HIV infection comprises administering an effective amount of the above mentioned composition to a host in need of protection or treatment against HIV to kill or inhibit HIV infection.

A deposit of plasmids pCD4ITM10G, pCD4ITM20G, pCD4ITM30G and pCD4ITM40G for the production of recombinant hybrid proteins in accordance with the present invention have been made at the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, 20852, U.S.A., on April 27, 1989 under accession numbers 67940, 67941, 67942 and 67943, respectively. The deposit shall be viably maintained, replacing if it becomes non-viable during the life of the patent, for a period of 30 years from the date of the deposit, or for 5 years from the last date of request for a sample of the deposit, whichever is longer, and made available to the public without restriction in accordance with the provisions of the law. The Commissioner of Patents and Trademarks, upon request shall have access to the deposit.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

- 48 -

WHAT IS CLAIMED IS:

1. A chimeric gene which directs the synthesis, in a suitable expression vector, of a hybrid protein comprising a virus binding region from a cellular receptor sequence linked to a protein toxin sequence containing a region essential for cell toxicity.
2. The gene of claim 1, wherein the cellular receptor sequence is from CD4 and the protein toxin sequence is from Pseudomonas exotoxin A.
3. The gene of claim 2 directing the synthesis of CD4(178)-PE40 fusion protein.
4. The gene of claim 3 inserted in a cloning vector.
5. The gene of claim 4 wherein said cloning vector has the functional characteristics of ATCC deposit No. 67739.
6. A cytotoxic agent comprising a hybrid protein comprising a virus binding region from a cellular receptor sequence linked to a protein toxin sequence containing a region essential for cell toxicity.
7. A cytotoxic agent of claim 6 wherein the cellular receptor sequence is from CD4 and protein toxin sequence is from Pseudomonas exotoxin A.
8. The cytotoxic agent of claim 7 being CD4(178)-PE40 recombinant fusion protein.
9. A recombinant molecule, comprising the N-terminal 177 amino acid residues of the mature human CD4, including the first two immunoglobulin type domains, which binds to gp120, the external subunit of human immunodeficiency virus envelope glycoprotein.
10. Hybrid protein, comprising gp120 binding region of human CD4 recombinantly linked to constant region of human IgG.
11. The hybrid protein of claim 10 being multimeric.
12. The hybrid protein of claim 11 being dimeric.

- 49 -

13. The hybrid protein of claim 11 being heterotetrameric composed of coexpressed CD4-human heavy chain constant region and normal human IgG light chains.

5 14. The hybrid protein of claim 11 being heterotetrameric composed of coexpressed CD4-human heavy chain constant region constructs coexpressed with CD4-human light chain constant region constructs.

15 15. The hybrid protein of claim 11 having binding affinity for complement component Clq.

10 16. The hybrid protein of claim 11 mediating complement dependent lysis of HIV infected cells expressing the HIV envelope glycoprotein.

17. The hybrid protein of claim 11 mediating complement dependent lysis of free HIV virions.

15 18. The hybrid protein of claim 11 mediating antibody-dependent cellular cytotoxicity.

19. The hybrid protein of claim 11 having binding affinity for Fc receptors on cells mediating antibody-dependent cellular cytotoxicity.

20 20. A composition, comprising effective amount of the cytotoxic agent of claim 6 to prevent proliferation of virus infection, and pharmaceutically acceptable, sterile, non-toxic carrier, if necessary.

25 21. The composition of claim 20 for preventing proliferation of HIV infection.

22. A composition comprising an effective amount of the polypeptide of claim 9 to inhibit binding of HIV to host cells.

30 23. A composition comprising an effective amount of the hybrid protein of claim 10 to inhibit HIV infection or kill HIV infected cells and virions and pharmaceutically acceptable carrier.

24. A method for controlling viral infection, comprising contacting virus-infected cells with cytotoxic amount of the agent of claim 6 to kill virus-infected cells.

25. A method for controlling HIV infection,

- 50 -

comprising contacting HIV-infected cells with cytotoxic amount of the agent of claim 6 to kill HIV-infected cells.

5 26. A method for preventing HIV infection of host cells, comprising providing an effective amount of the polypeptide of claim 9 to host cells to inhibit interaction between gp120 and natural CD4 on the host cells.

10 27. A method of controlling HIV infection, comprising contacting HIV infected cells or virions with an effective amount of the composition of claim 23 to selectively kill HIV infected cells or inhibit HIV activity.

1 / 2 4

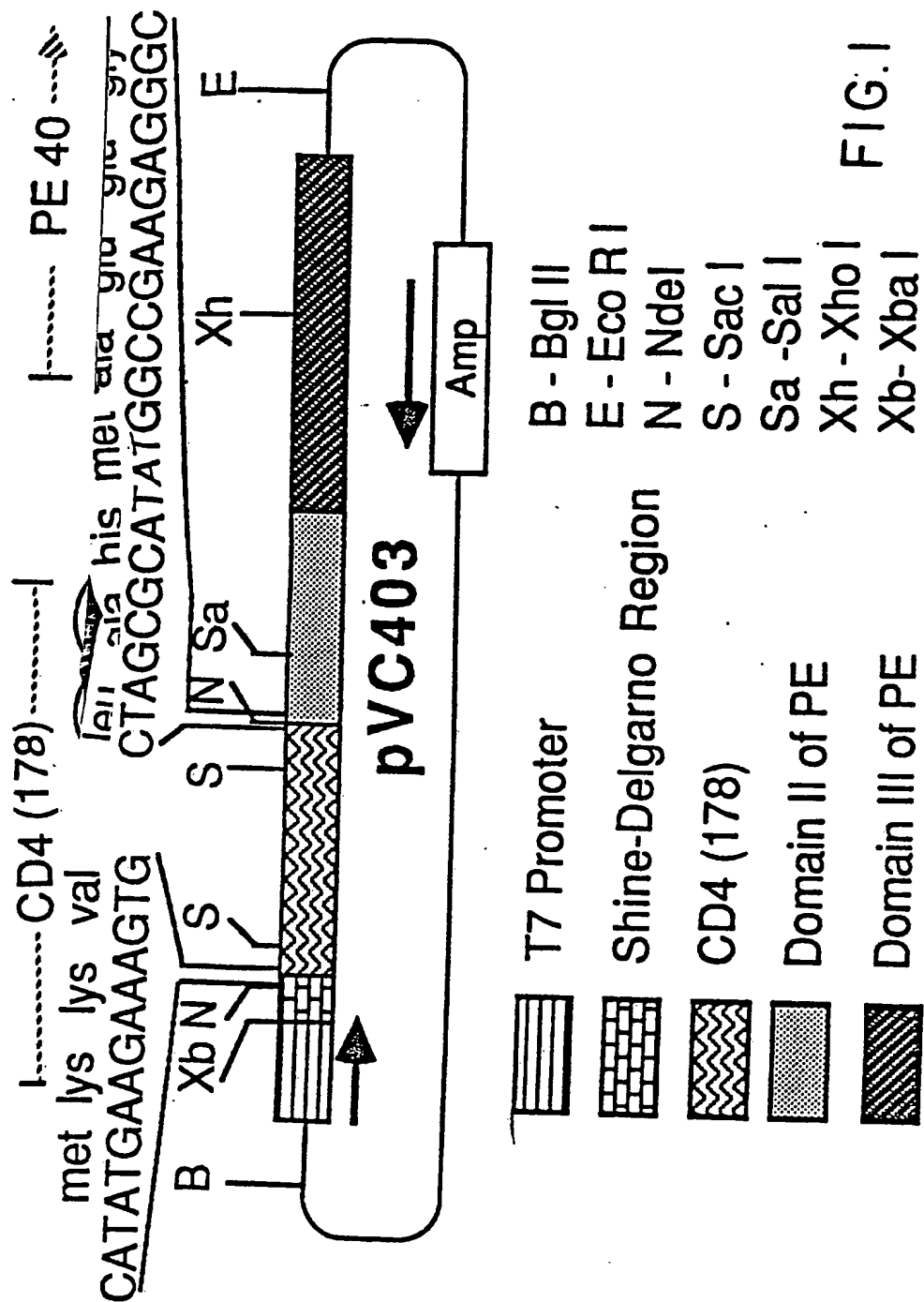


FIG. 1

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2 / 24

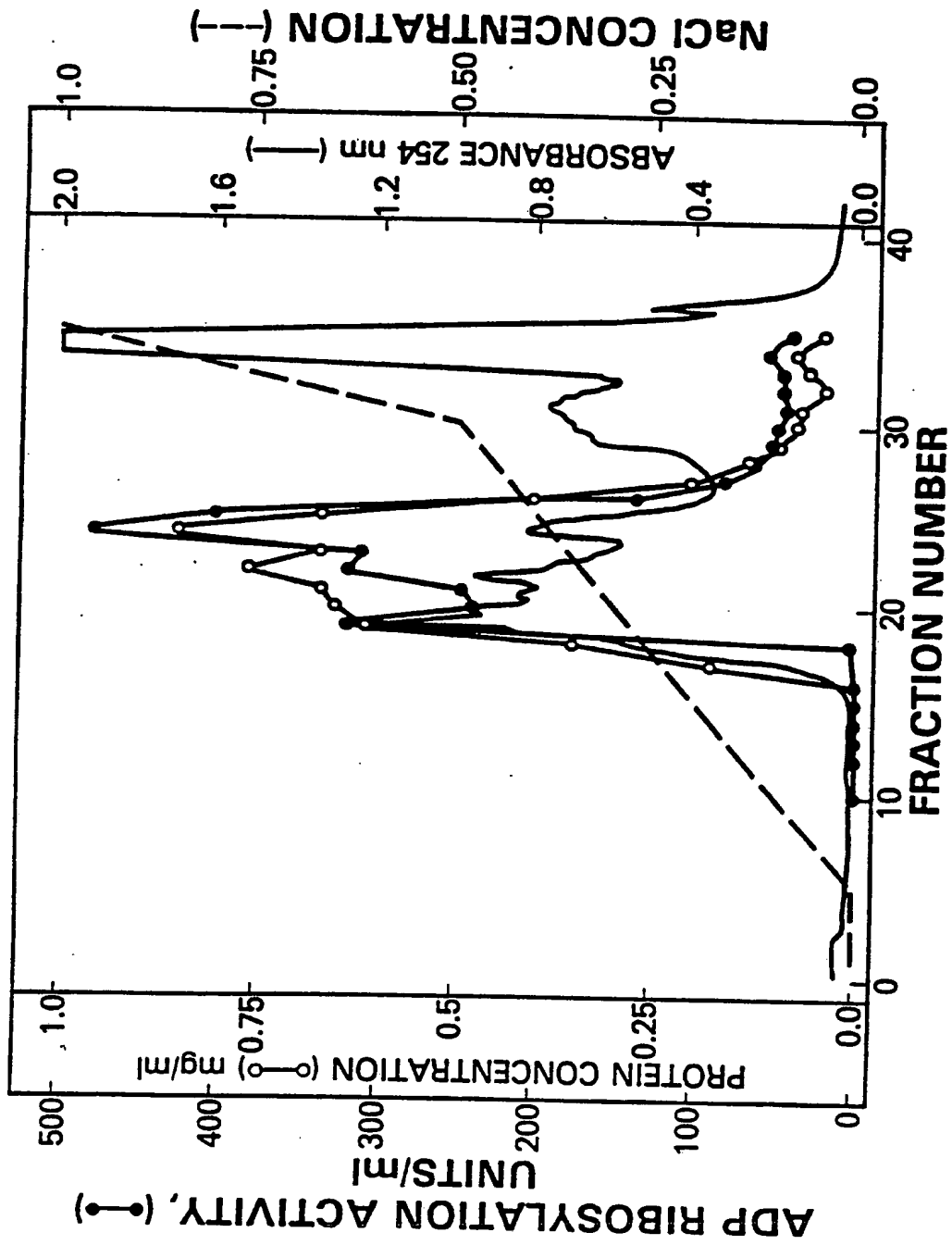
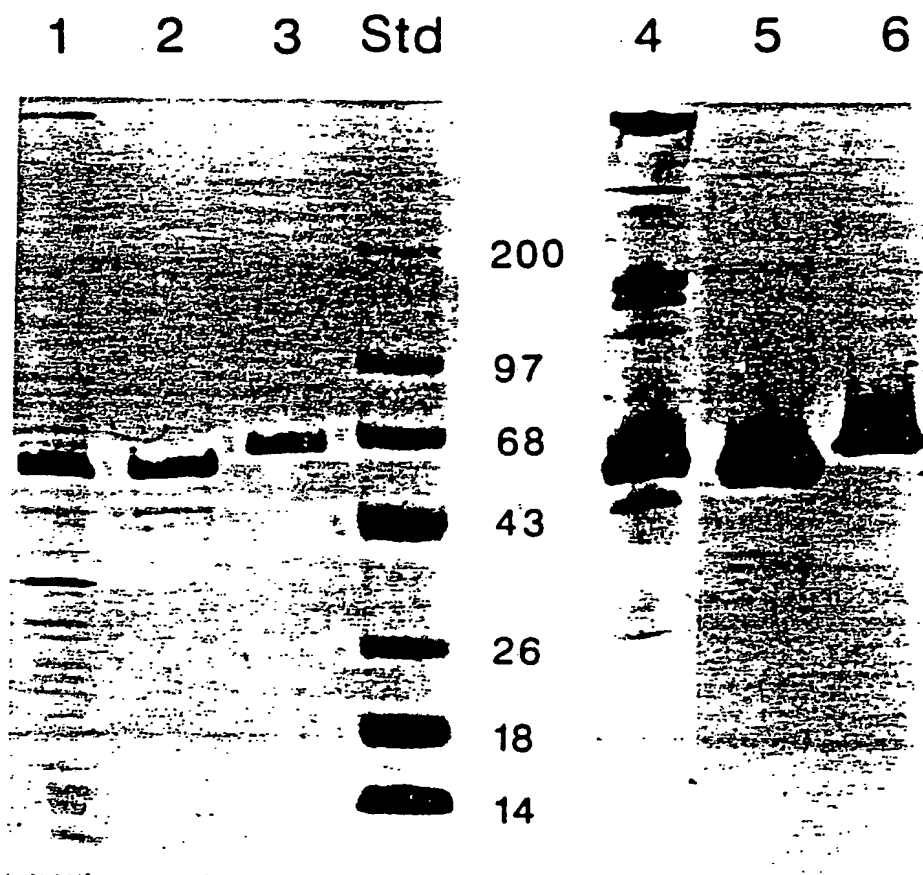


FIG.2A

SUBSTITUTE SHEET

3 / 2 4

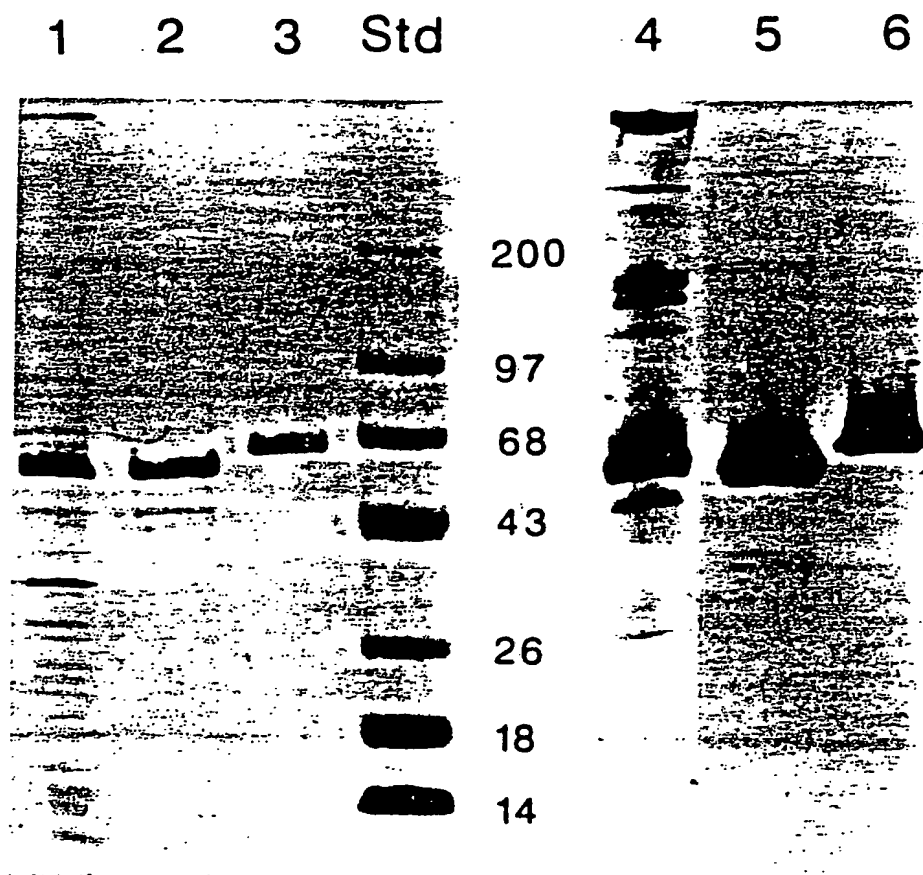
FIG. 2B



SUBSTITUTE SHEET

3 / 24

FIG.2B



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4 / 24

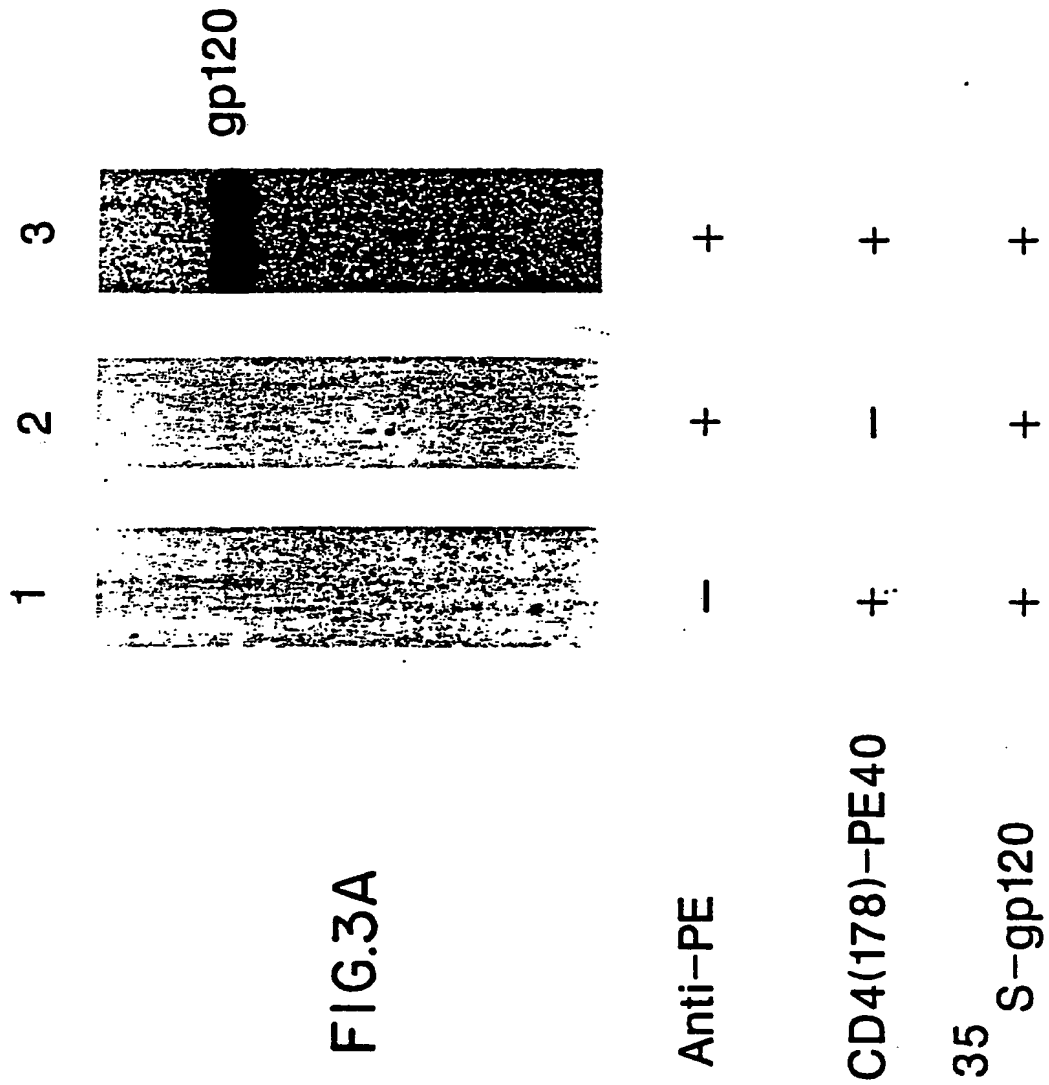
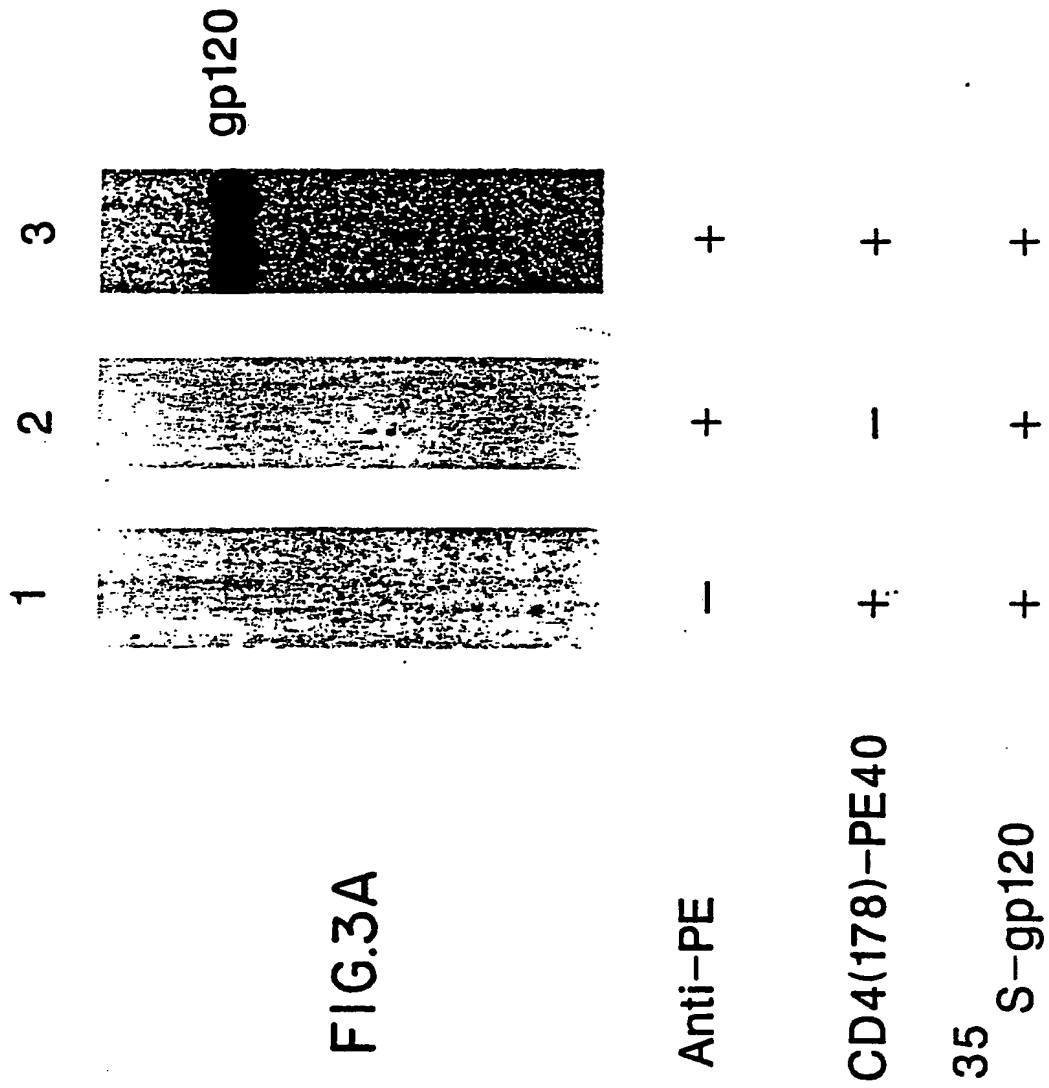


FIG.3A



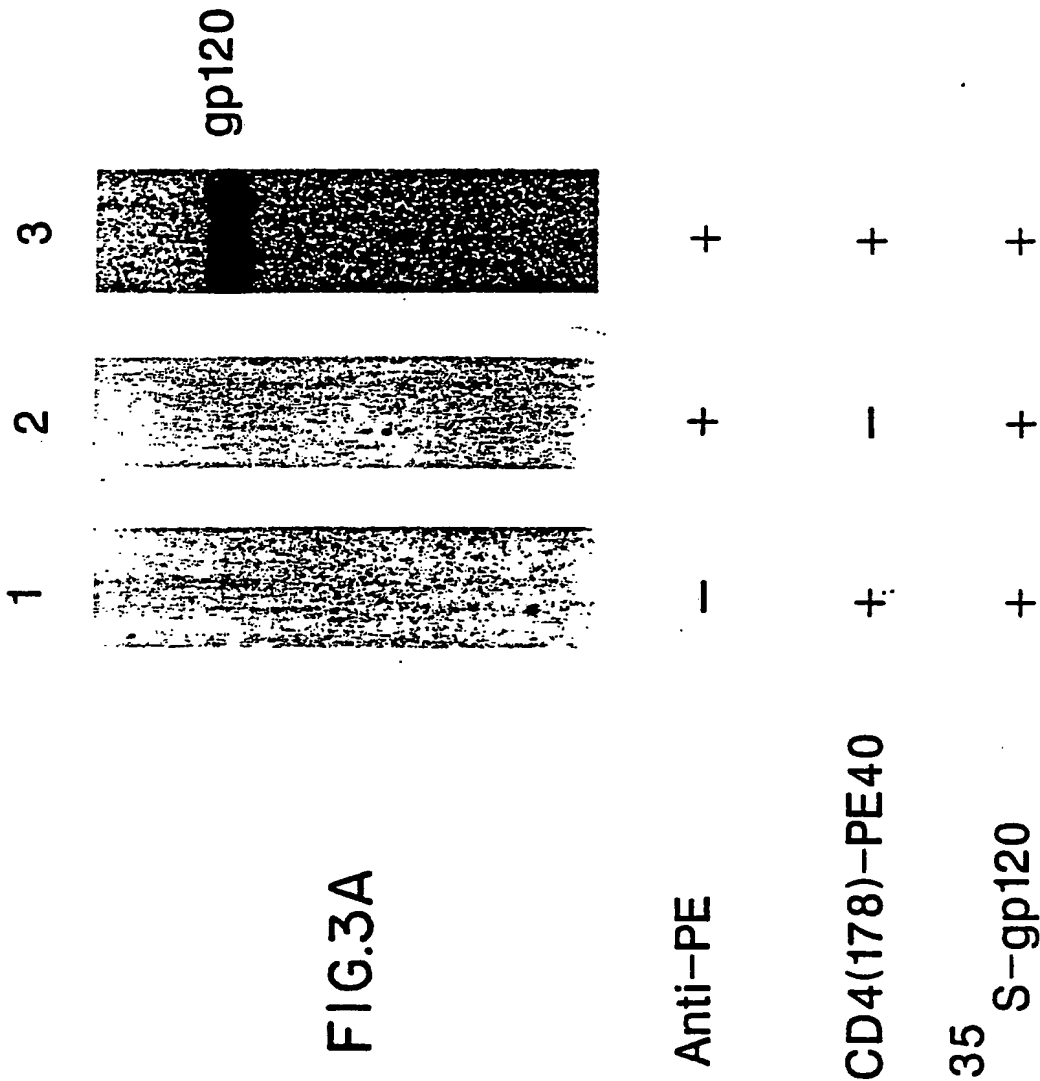


FIG.3A

5 / 24

FIG.3C

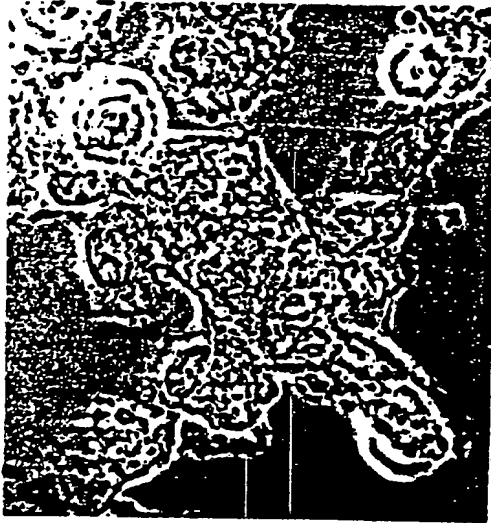


FIG.3E



FIG.3B

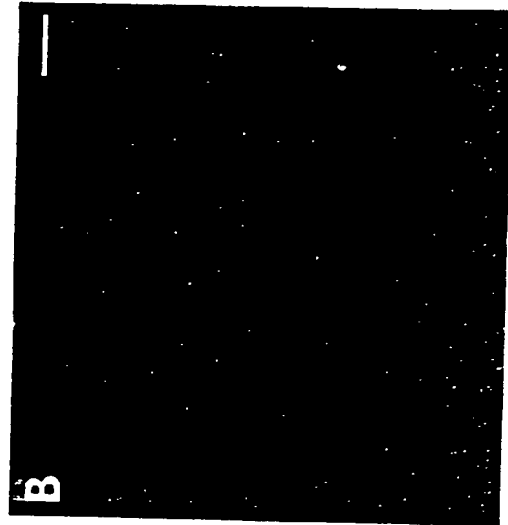


FIG.3D

6 / 24

FIG.4B

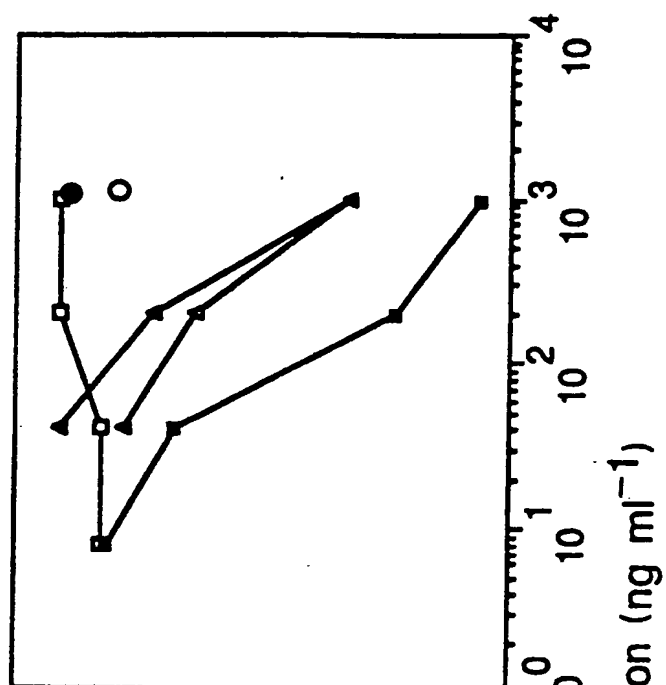
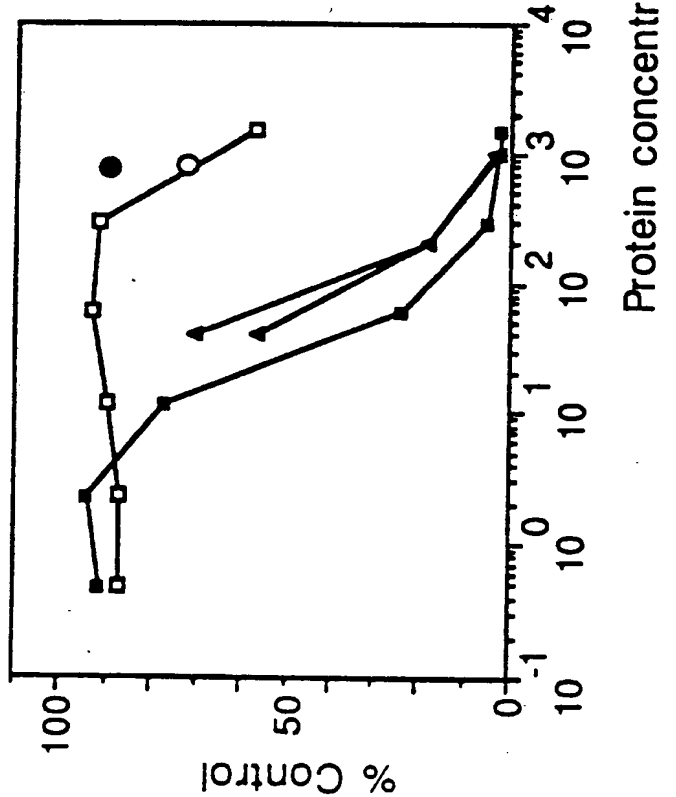


FIG.4A



SUBSTITUTE SHEET

7 / 24

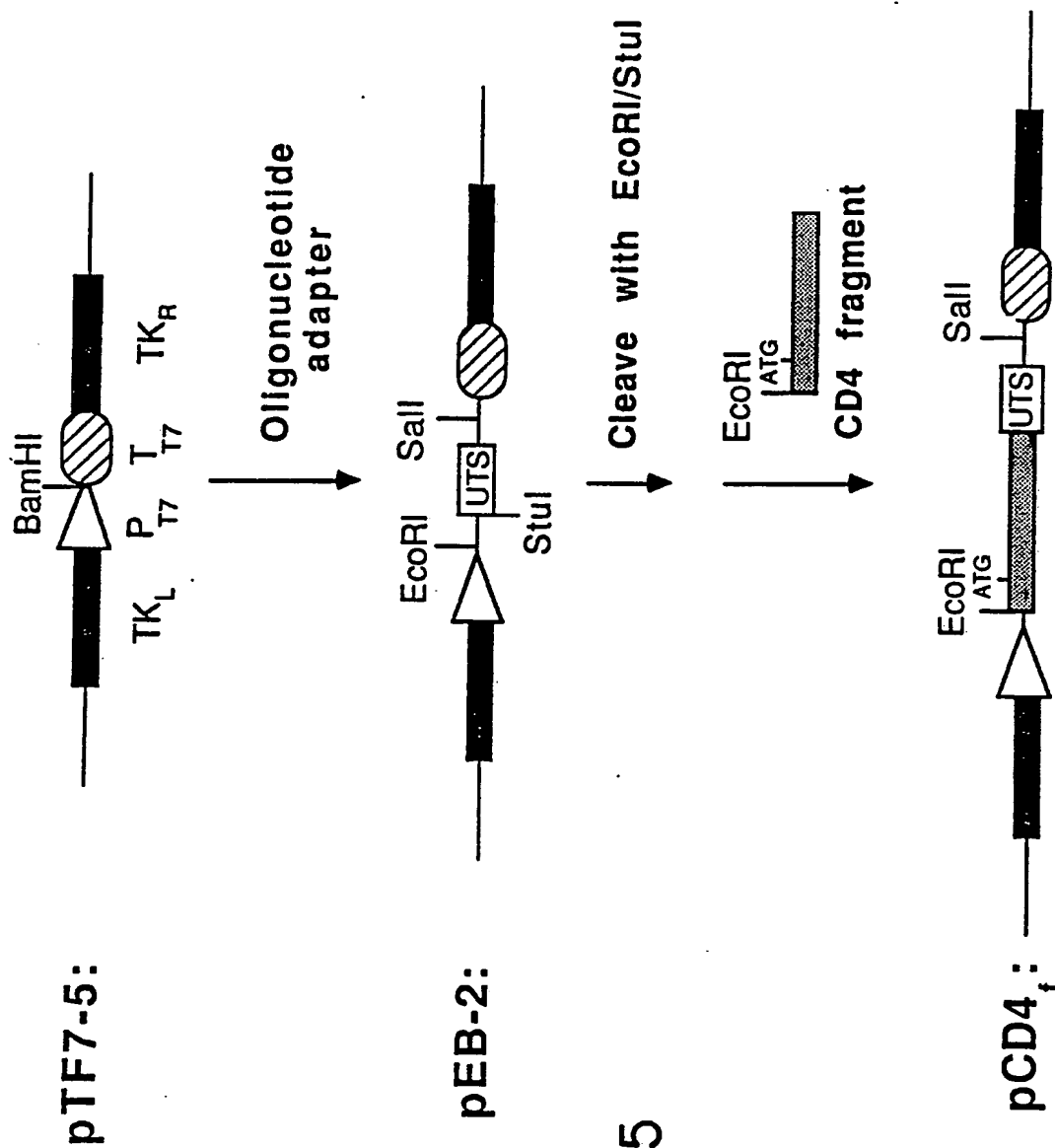


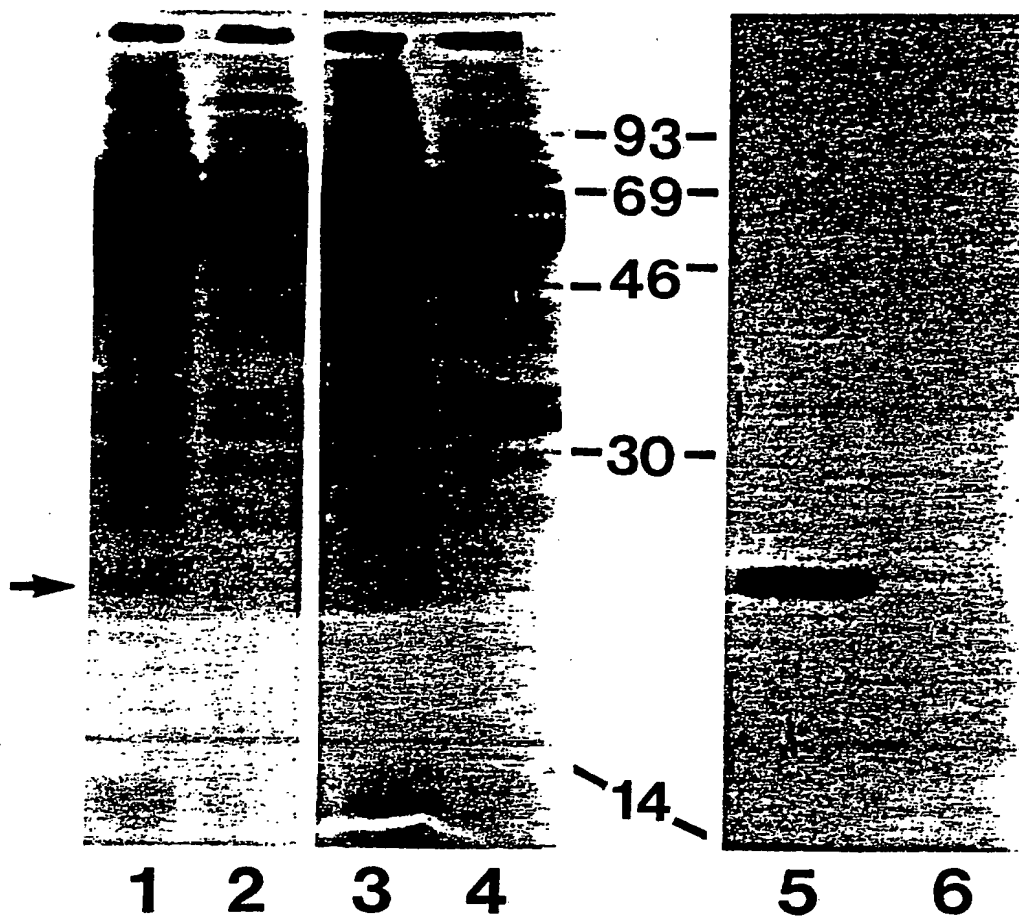
FIG.5

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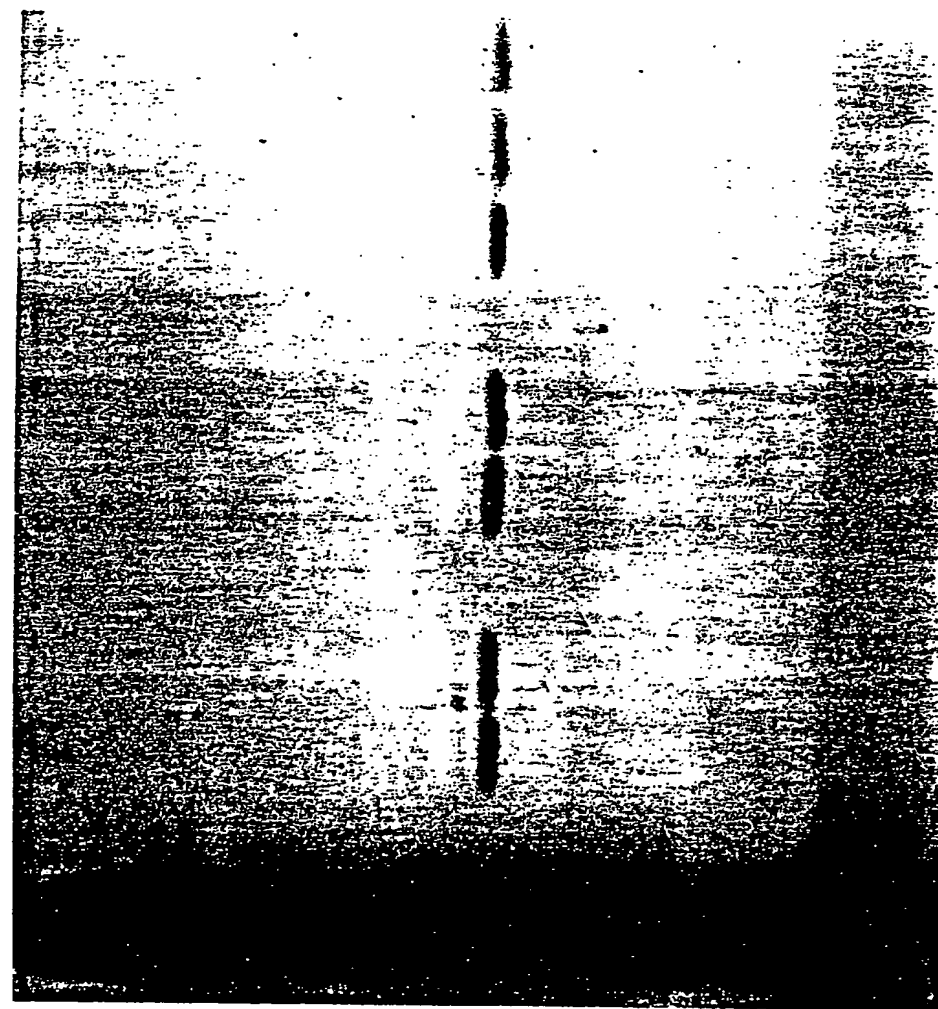
8 / 24

FIG.6A

FIG.6B



9 / 24



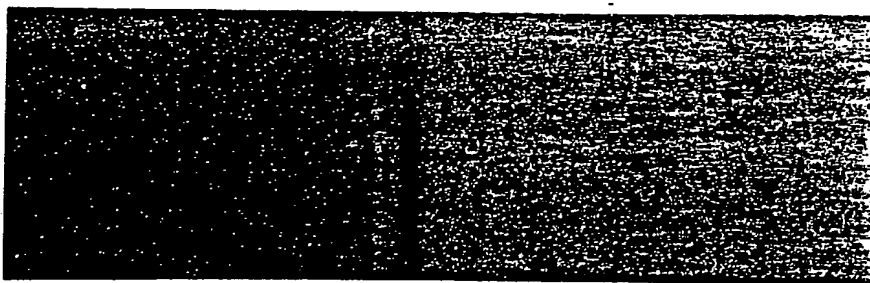
200-
93-
69-
46-
30-
14-

FIG.7

1 2 3 4 5 6 7 8 9 10 11

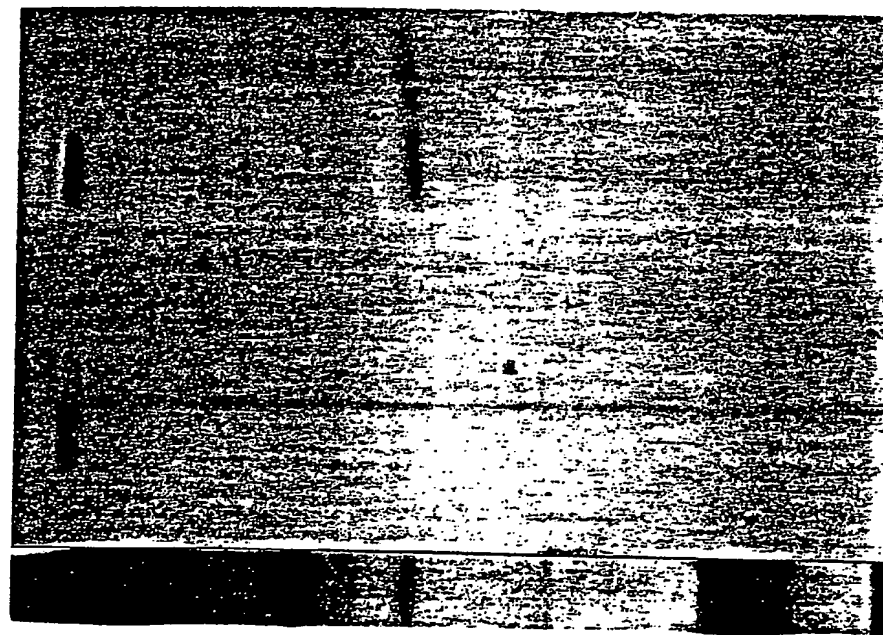
10 / 24

FIG.8B



8 9 10

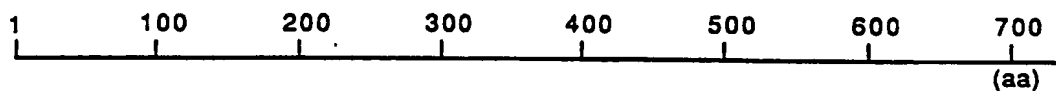
FIG.8A



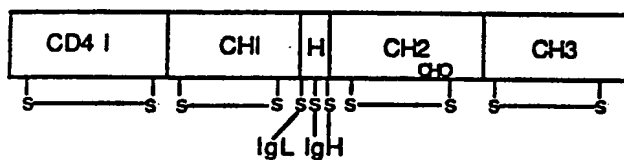
1 2 3 4 5 6 7

200-
93-
69-
46-
30-
14-

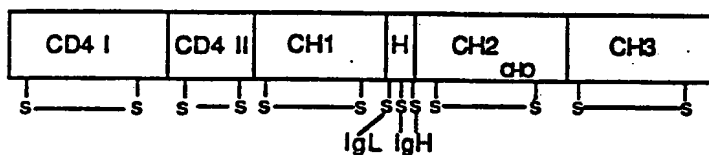
11 / 24



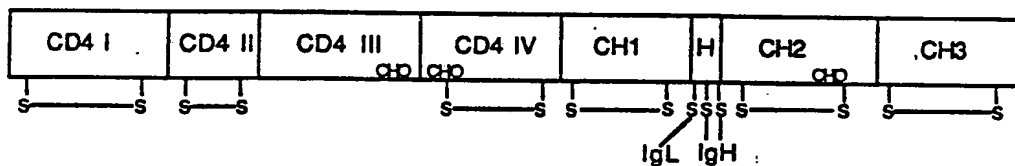
CD4(109)CH; 439aa (pCD4ITM10G)



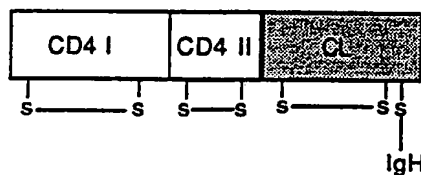
CD4(178)CH; 508aa (pCD4ITM20G)



CD4(372)CH; 702aa (pCD4ITM30G)



CD4(181)CL; 292aa (pCD4ITM40G)

**KEY**

- CD4 Extracellular Domains
- IgG1 Heavy Chain Constant Domains
- Ig Light Chain Constant Domain

FIG.9

SUBSTITUTE SHEET

12/24

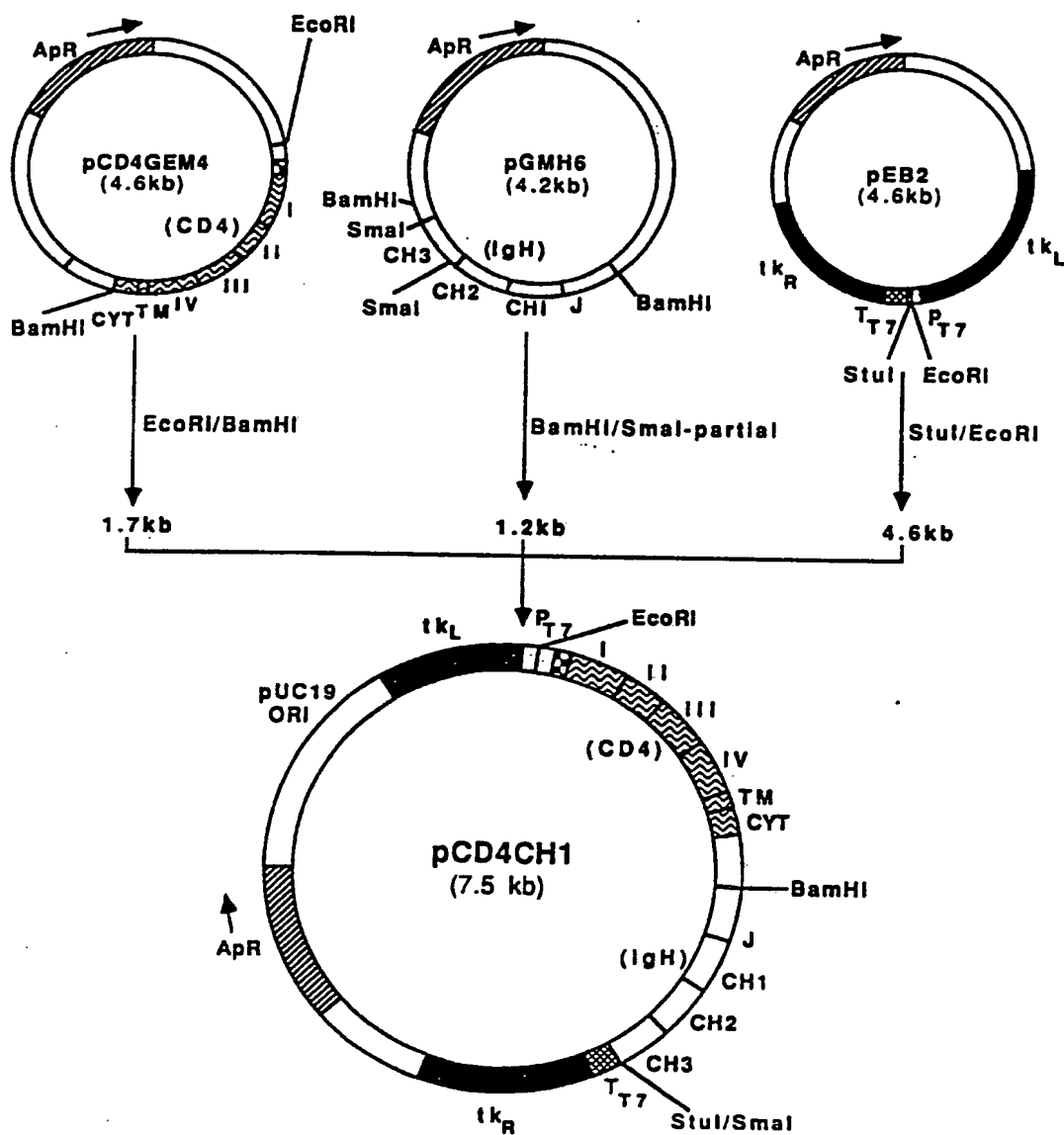


FIG.10

SUBSTITUTE SHEET

13 / 24

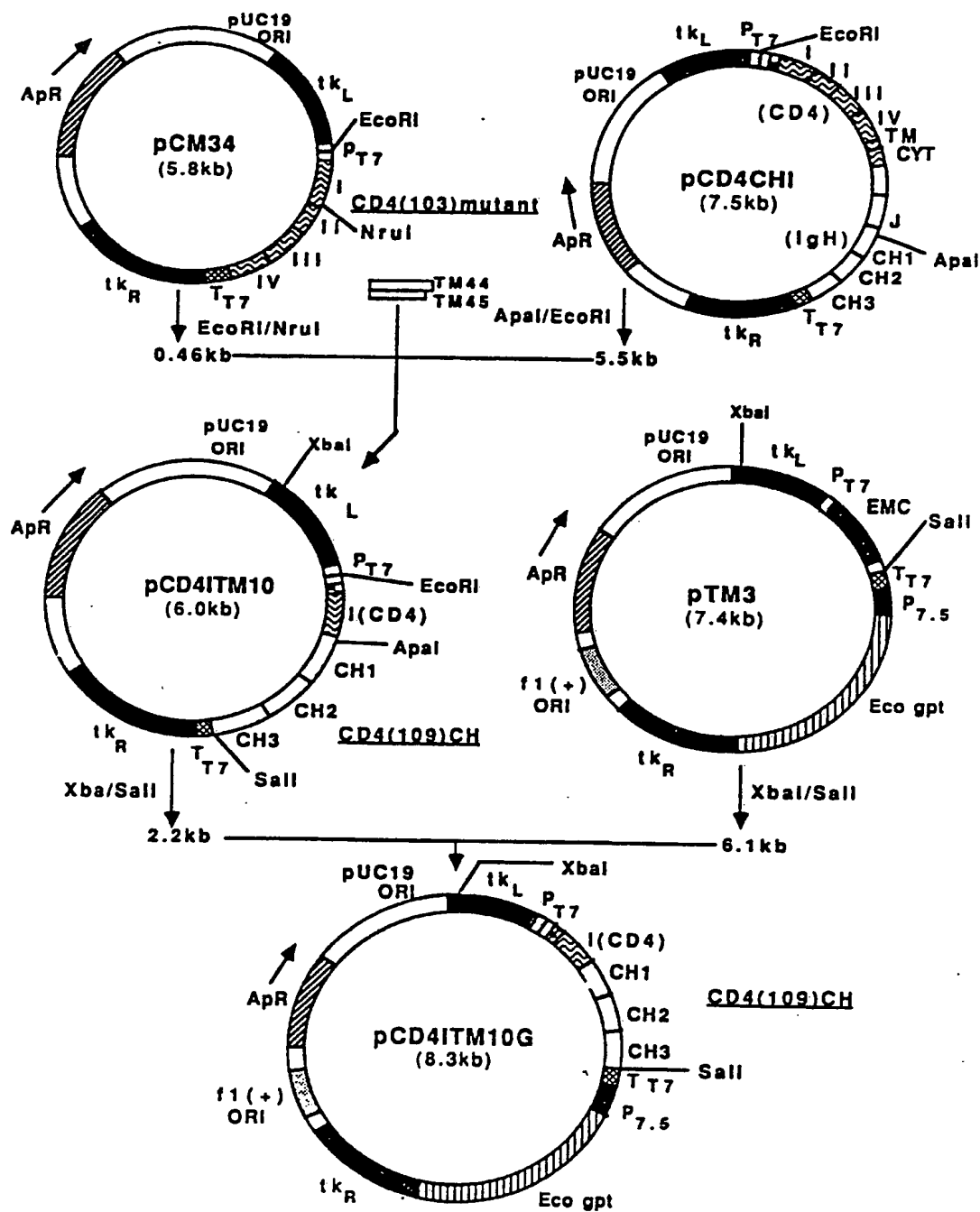


FIG. II

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14 / 24

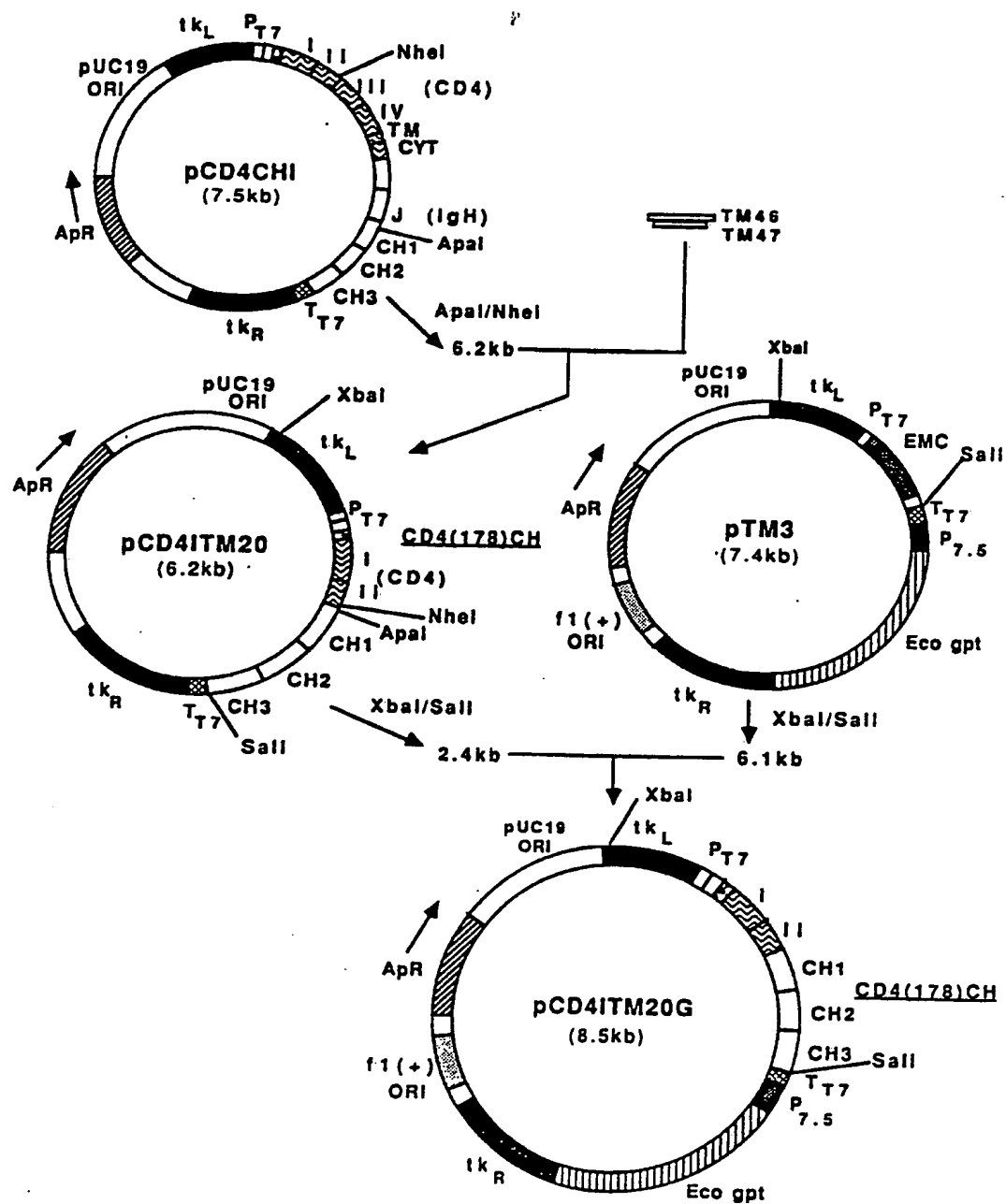


FIG.12

SUBSTITUTE SHEET

15 / 24

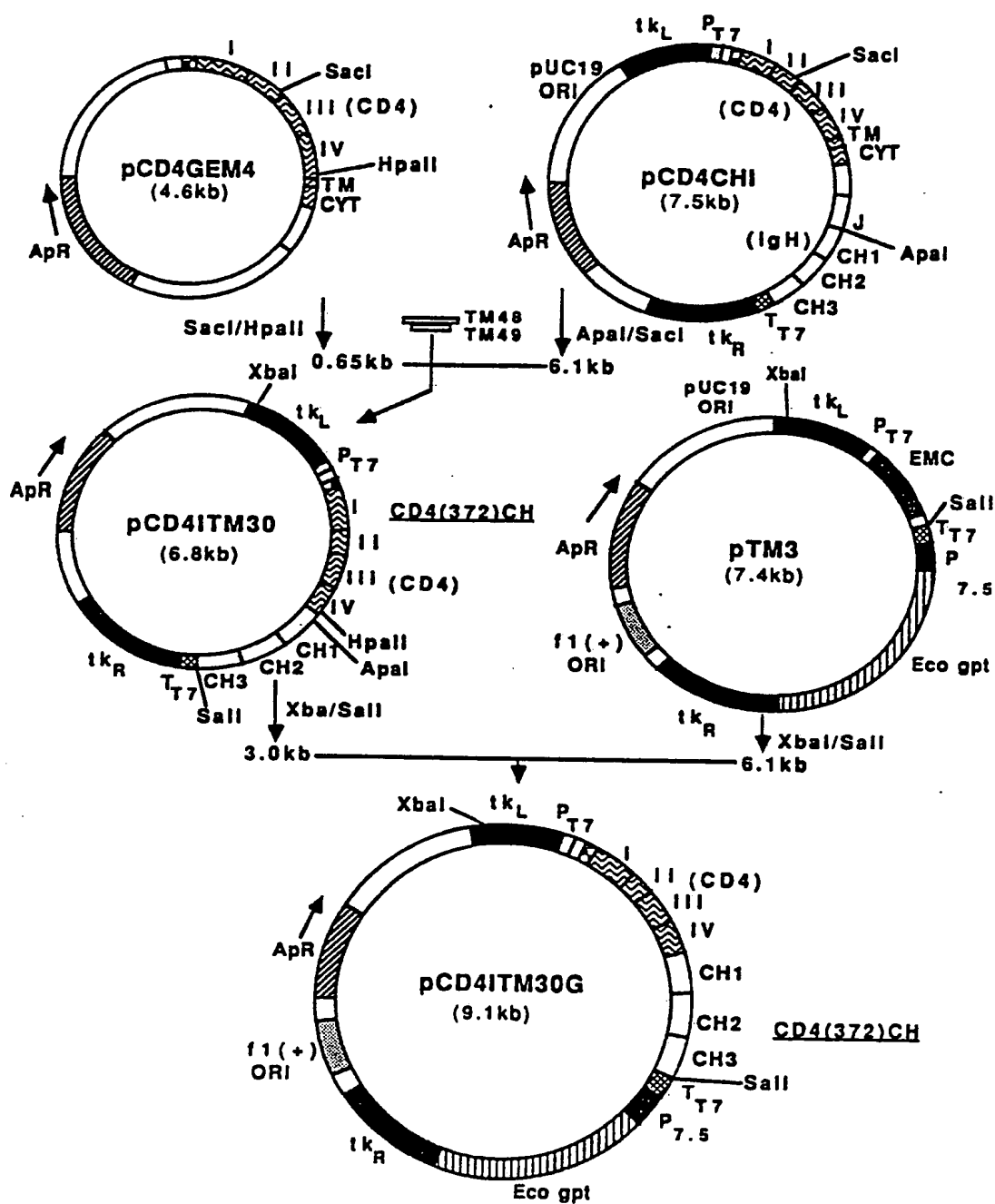


FIG.13

SUBSTITUTE SHEET

16 / 24

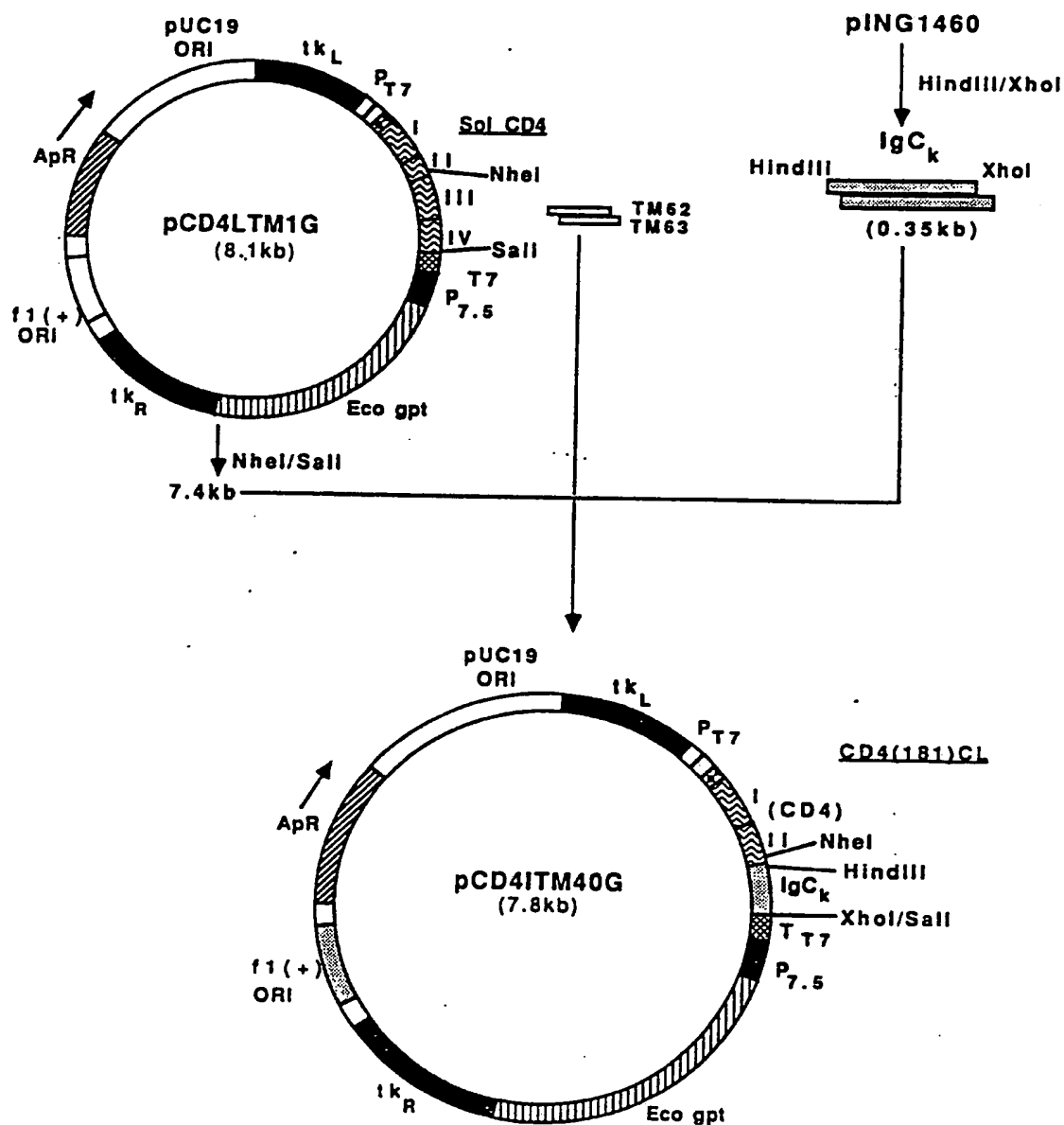
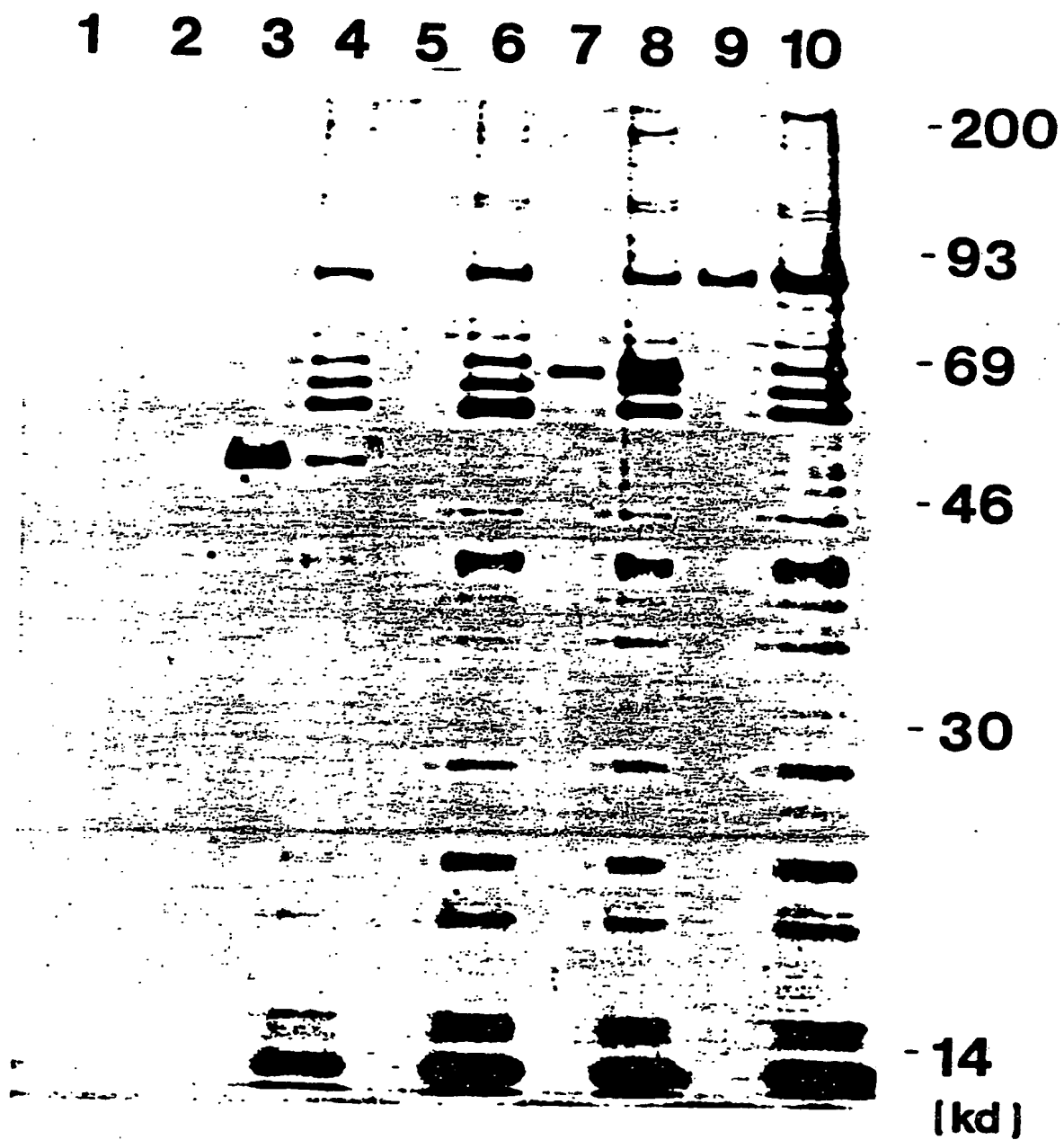


FIG.14

SUBSTITUTE SHEET

17/24

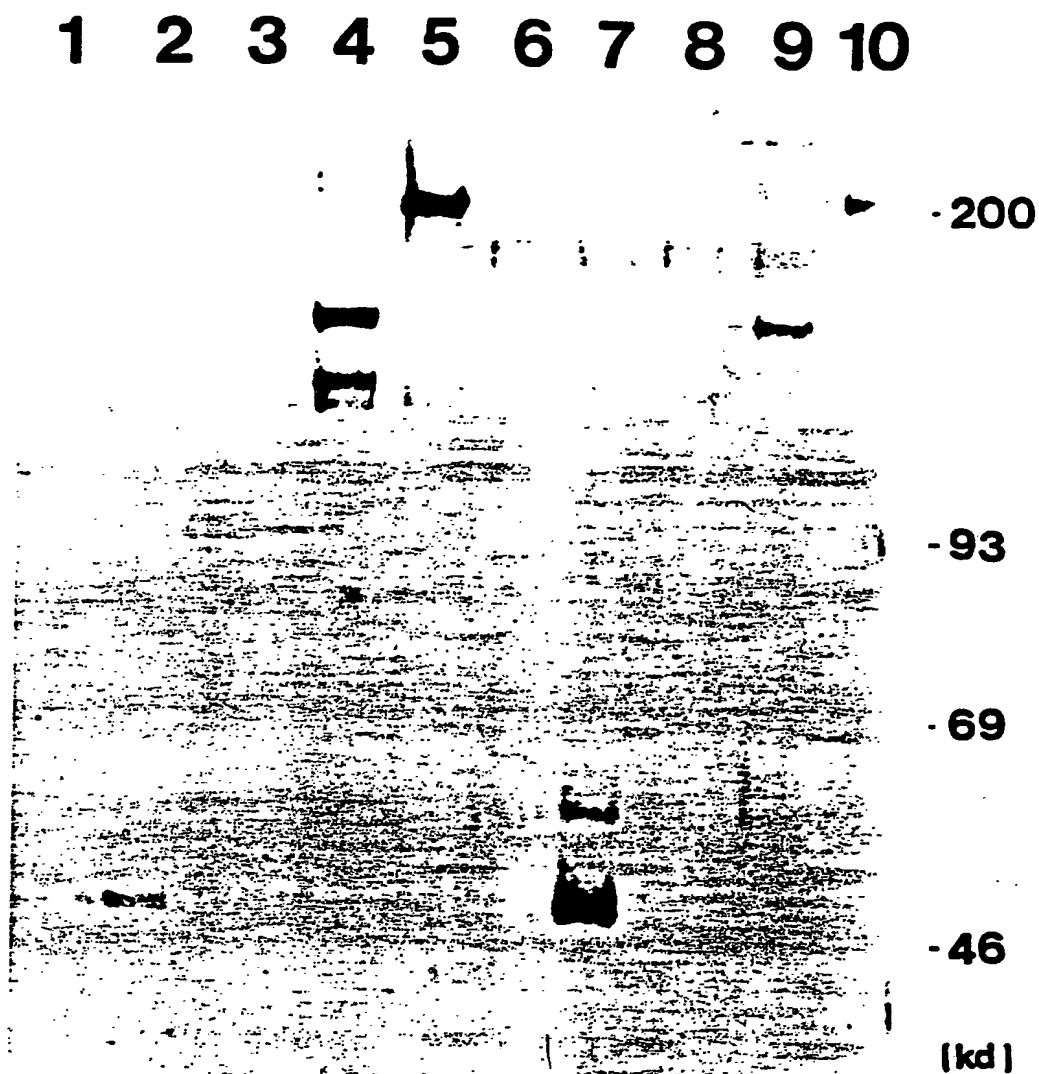
FIG.15



SUBSTITUTE SHEET

18/24

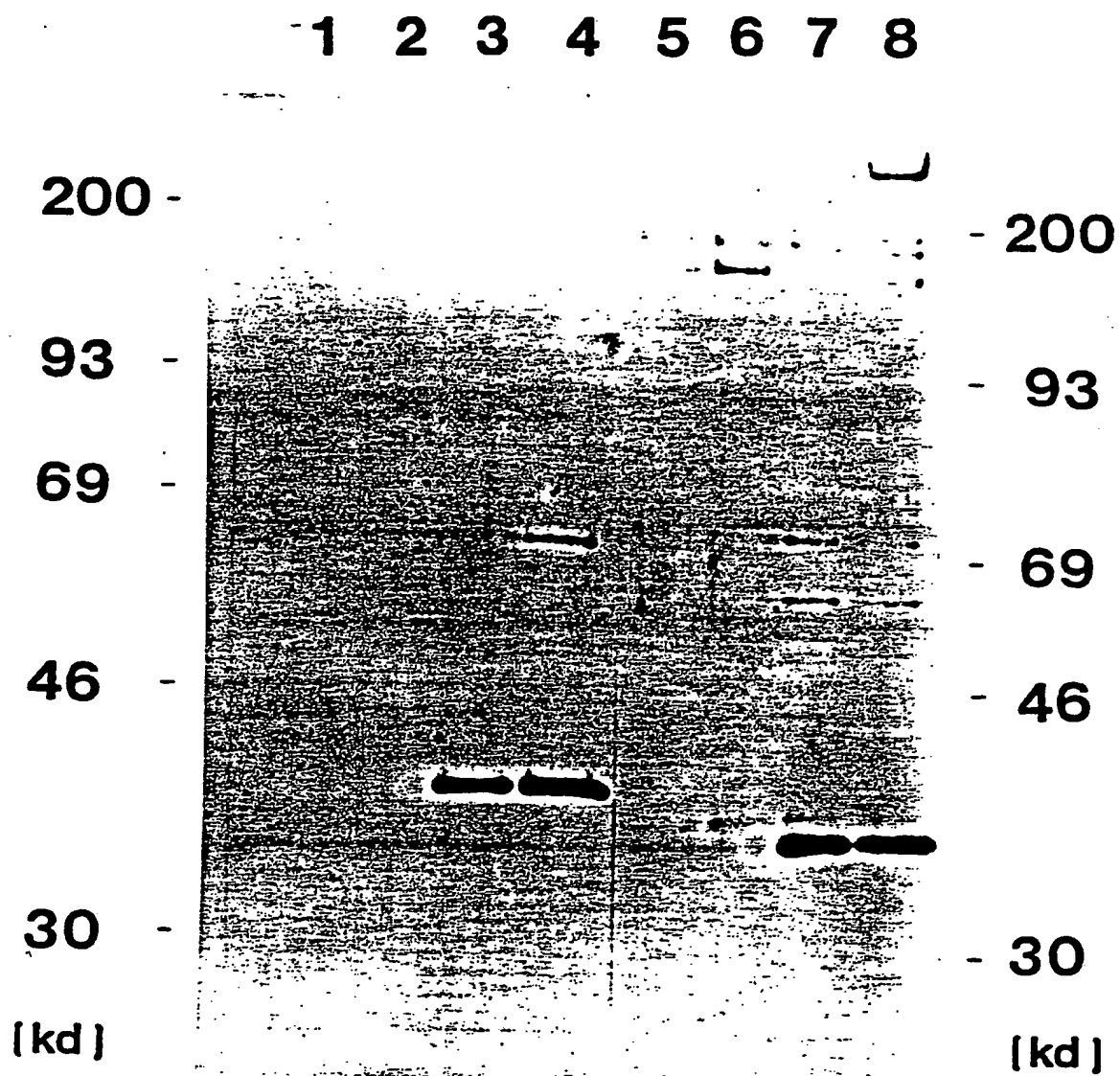
FIG.16



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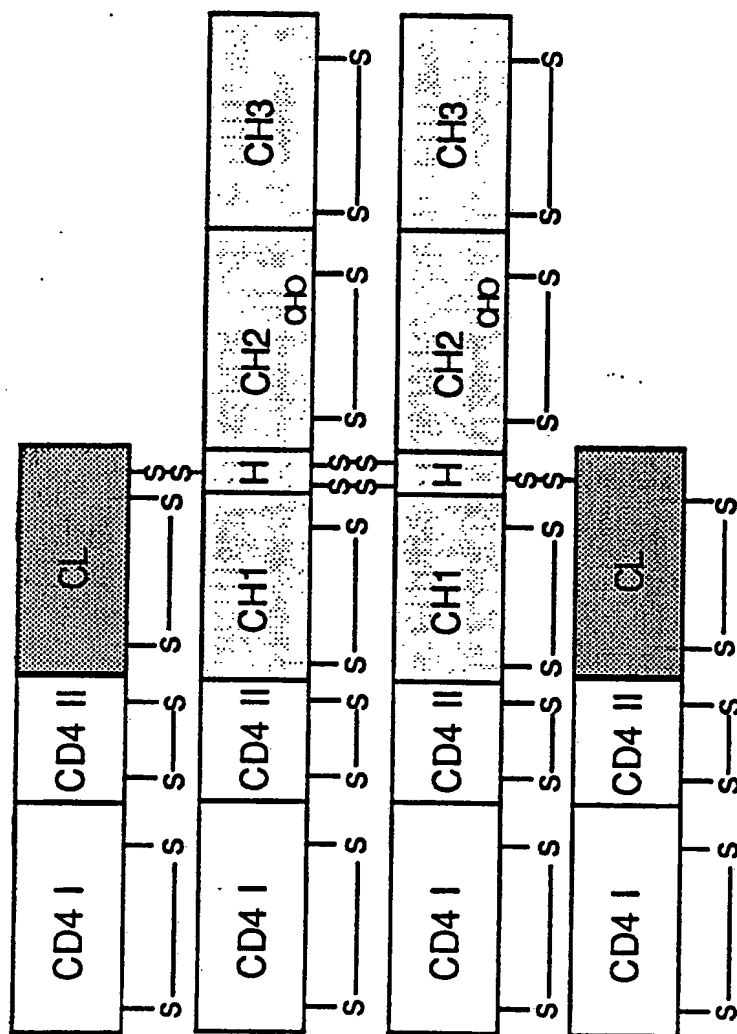
19/24

FIG.17



SUBSTITUTE SHEET

20 / 24



KEY




-  CD4 Extracellular Domains
-  IgG1 Heavy Chain Constant Domains
-  Ig Light Chain Constant Domain

FIG.18

SUBSTITUTE SHEET

21 / 24

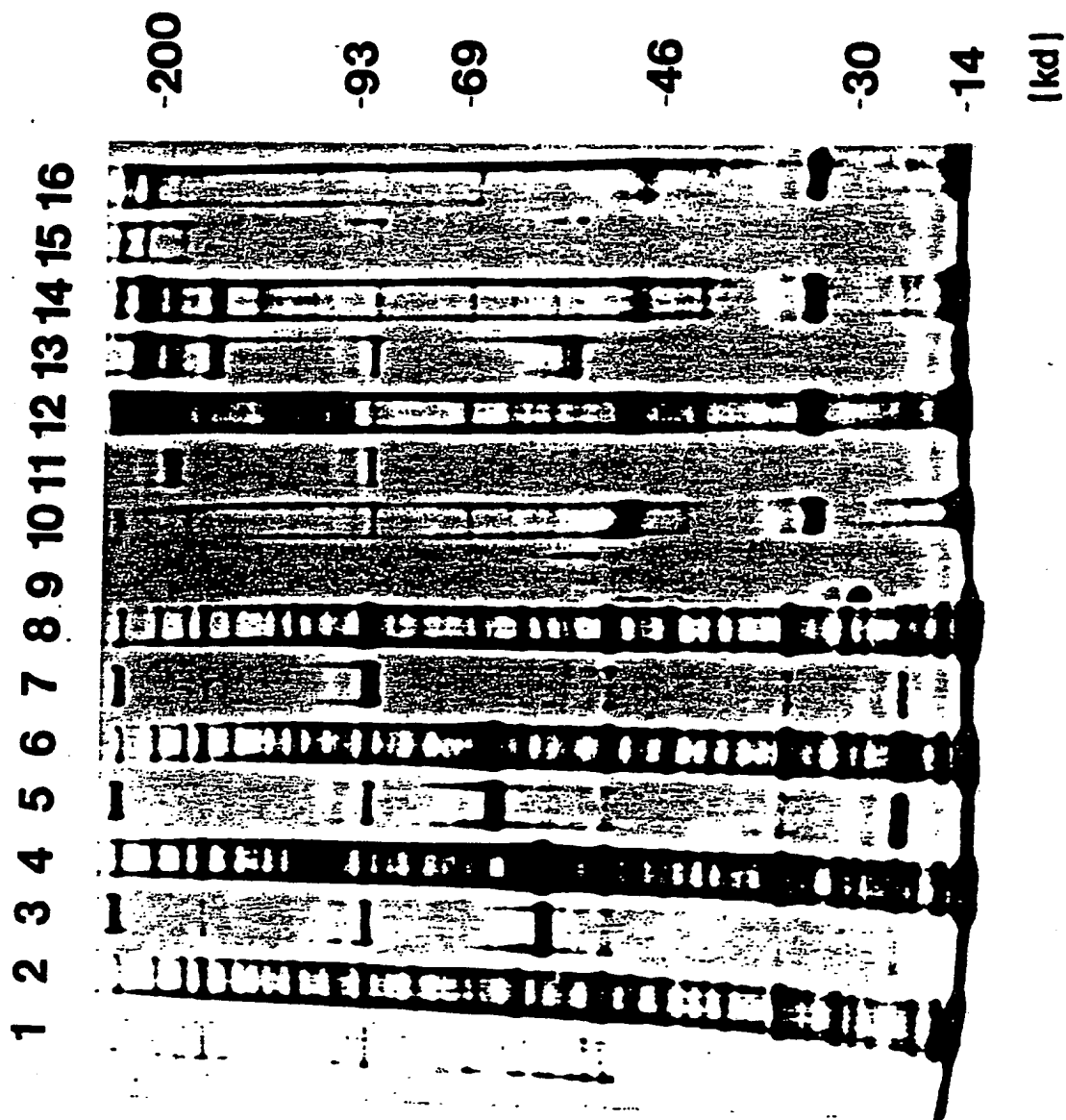
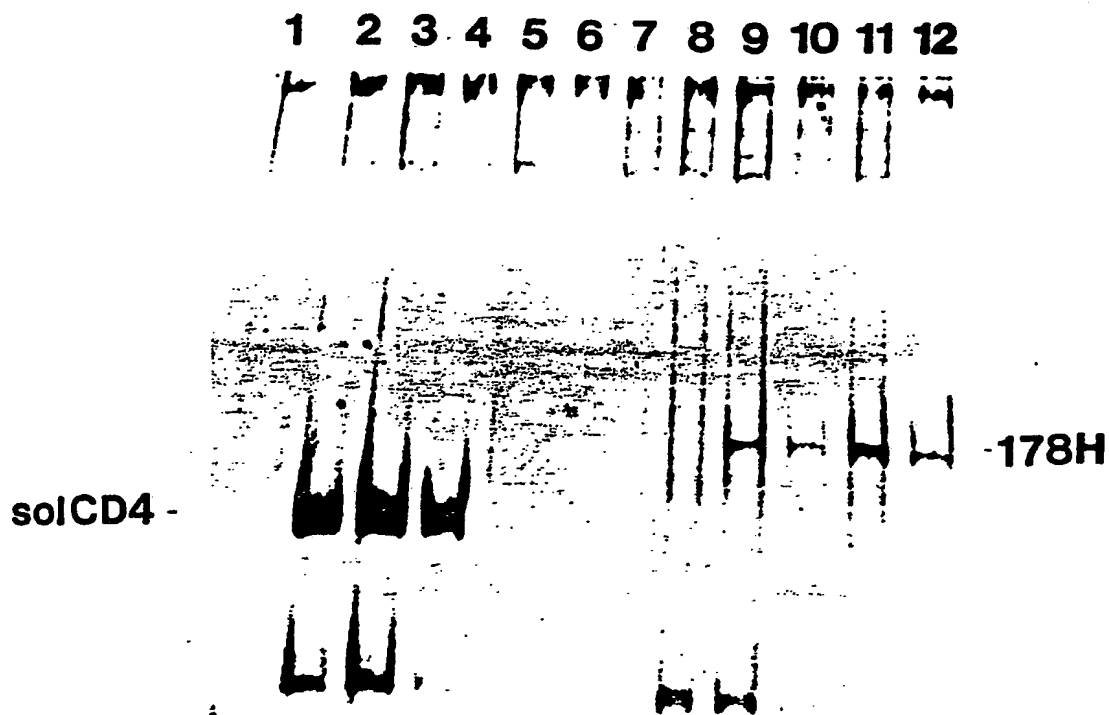


FIG.19

SUBSTITUTE SHEET

22 / 24

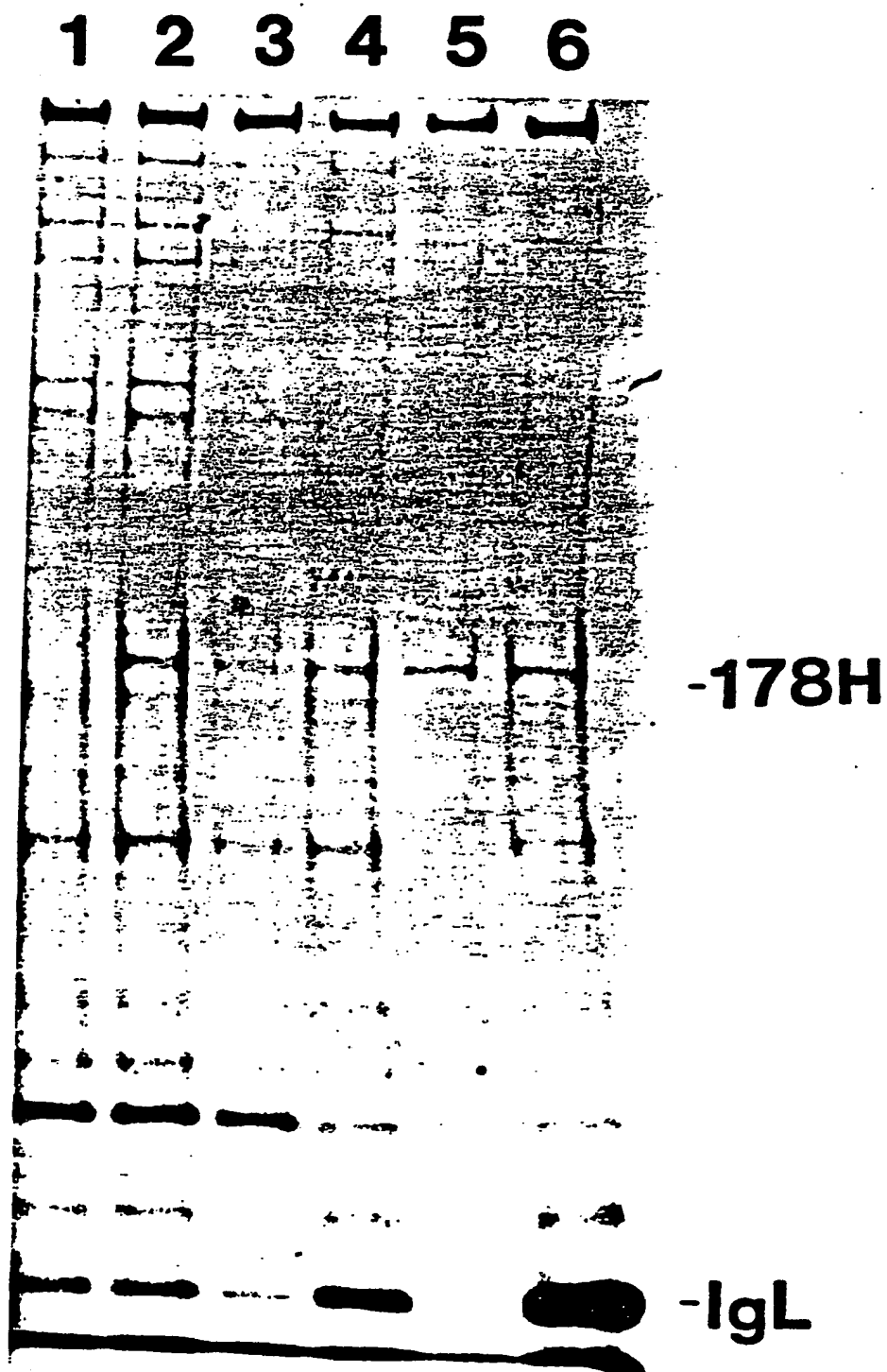
FIG.20



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23/24

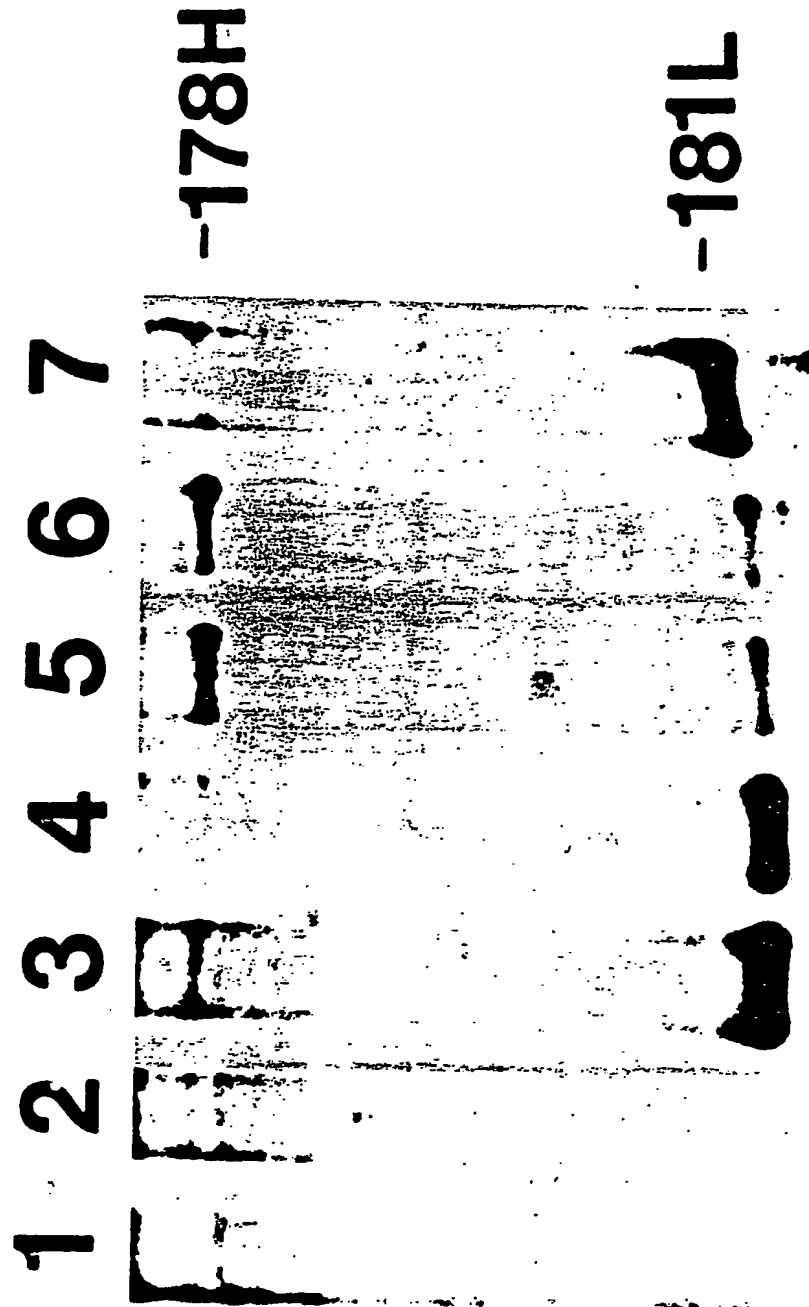
FIG. 21



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24/24

FIG. 22



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INTERNATIONAL SEARCH REPORT

PCT/US89/03267

International Application No.

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC(4): C 07 H 15/12; C 07 K 13/00; A 61 K 37/48, 39/00														
II. FIELDS SEARCHED <div style="text-align: center; font-size: small;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border: none;"> <tr> <td style="width: 30%; border: 1px solid black; padding: 5px;">Classification System</td> <td style="border: none;"></td> <td style="width: 70%; border: 1px solid black; padding: 5px;">Classification Symbols</td> </tr> <tr> <td style="border: 1px solid black; padding: 5px;">U.S.</td> <td style="border: none;"></td> <td style="border: 1px solid black; padding: 5px;">424/88; 514/8; 530/387; 536/27</td> </tr> </table> <div style="text-align: center; font-size: x-small; margin-top: 5px;">Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁸</div>			Classification System		Classification Symbols	U.S.		424/88; 514/8; 530/387; 536/27						
Classification System		Classification Symbols												
U.S.		424/88; 514/8; 530/387; 536/27												
CA, Biosis 1967-1989														
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; font-size: x-small;">Category ¹⁰</th> <th style="width: 70%; font-size: x-small;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 20%; font-size: x-small;">Relevant to Claim No. ¹³</th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td>Science, Volume 231, published 24 January 1986. J.S. McDougal, et al. "Binding of HTLV-III/LAV to T4+ cells by a complex of the 110K viral protein and the T4 molecule" pp. 382-385. See entire article.</td> <td style="text-align: center; vertical-align: top;">1-27</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td>Proc. Natl. Acad. Sci. USA, Volume 84, published July 1987. V.K. Chaudhary, et al. "Activity of a recombinant fusion protein between transforming growth factor type alpha and Pseudomonas toxin. pp. 4538-4542. see entire article.</td> <td style="text-align: center; vertical-align: top;">1-27</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td>Cell, Volume 42, published August 1985. P.J. Maddon, et al. "The isolation and nucleotide sequence of a cDNA encoding the T cell surface protein T4; a new member of the immunoglobulin gene family. pp93-104. see entire article.</td> <td style="text-align: center; vertical-align: top;">1-27</td> </tr> </tbody> </table>			Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	Y	Science, Volume 231, published 24 January 1986. J.S. McDougal, et al. "Binding of HTLV-III/LAV to T4+ cells by a complex of the 110K viral protein and the T4 molecule" pp. 382-385. See entire article.	1-27	Y	Proc. Natl. Acad. Sci. USA, Volume 84, published July 1987. V.K. Chaudhary, et al. "Activity of a recombinant fusion protein between transforming growth factor type alpha and Pseudomonas toxin. pp. 4538-4542. see entire article.	1-27	Y	Cell, Volume 42, published August 1985. P.J. Maddon, et al. "The isolation and nucleotide sequence of a cDNA encoding the T cell surface protein T4; a new member of the immunoglobulin gene family. pp93-104. see entire article.	1-27
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<div style="display: flex; justify-content: space-between; font-size: x-small;"> <div style="width: 45%;"> <p>* Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>														
IV. CERTIFICATION <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none;"> Date of the Actual Completion of the International Search <div style="text-align: center; font-size: large;">October 30, 1989</div> </td> <td style="width: 50%; border: none;"> Date of Mailing of this International Search Report <div style="text-align: center; font-size: large;">28 NOV 1989</div> </td> </tr> <tr> <td style="border: none;"> International Searching Authority <div style="text-align: center; font-size: large;">ISA/US</div> </td> <td style="border: none;"> Signature of Authorized Officer <div style="text-align: center;"> Beth A. Burrous </div> </td> </tr> </table>			Date of the Actual Completion of the International Search <div style="text-align: center; font-size: large;">October 30, 1989</div>	Date of Mailing of this International Search Report <div style="text-align: center; font-size: large;">28 NOV 1989</div>	International Searching Authority <div style="text-align: center; font-size: large;">ISA/US</div>	Signature of Authorized Officer <div style="text-align: center;"> Beth A. Burrous </div>								
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International Searching Authority <div style="text-align: center; font-size: large;">ISA/US</div>	Signature of Authorized Officer <div style="text-align: center;"> Beth A. Burrous </div>													

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category	Citation of Document, with indication where appropriate, of the relevant passages	Relevant to Claim No.
Y	Proc. Natl. Acad. Sci. USA. Volume 81, published November 1984. S. Morrison, et al. "Chimeric human antibody molecules: mouse antigen-binding domains with human constant region domains" pp. 6851-6855, see entire article.	14,15
Y	Biotechniques. Volume 4, number 3. published 1986. V. Oi, et al. "Chimeric antibodies" pp. 214-220. see entire article.	14,15

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